INTRODUCTION

1.1 Background:

Primary teeth guide the eruption of permanent dentition and are important for jaw development, mastication, phonetics, appearance and psychological comfort. Primary teeth are thus an integral part of the general health, growth and development of children. Premature loss of teeth can produce problems in the eruption of succedaneous teeth, speech disturbances and harmful oral habits like tongue thrusting.¹

Preservation of primary tooth in a disinfected state is the best space maintainer.² Pulp therapy is the ideal treatment alternative but it has its own challenges. Pulp therapy in primary teeth is a subject of countless controversies, exhaustive literature and numerous techniques in an attempt to preserve the natural tooth. Some of the challenges involving pulp therapy in primary teeth is the complex root canal morphology with numerous ramifications and anatomical irregularities. The highly porous inter-radicular dentin at the pulpal floor allows pulpal infection to easily spread to the furcal region and can affect the developing permanent tooth bud.³ Some authors advocate the extraction of tooth and placement of space maintainers. Yet, these appliances have some disadvantages in maintaining oral hygiene and function too.⁴

Successful pulp therapy in primary teeth depends on reduction and elimination of root canal infection; although no techniques for root canal instrumentation have been followed.⁵ Necrotic primary teeth, especially of long-standing nature and symptomatic with periapical bone destruction tend to harbor a high bacterial load and more complex anaerobic bacterial flora.⁵,⁶ Intracanal medicaments are essential to reduce these virulent and pathogenic bacteria for successful pulp therapy.⁷ Success of
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Pulpectomized teeth depend on adequate disinfection of root canal systems before obturation of the primary tooth.\(^8\) Hence the use of intracanal medicaments may be required to resolve infection and promote healing.

Ideally an intracanal medicament should be able to neutralize the virulence of microorganisms and pathogenic factors (such as proteins, enzymes, toxins, aggregation substances) and induce a host response that favours periapical tissue healing. The continuous presence, however, of positive microbial cultures after root canal shaping, sanitization and use of calcium hydroxide as interappointment, intracanal dressing justifies the investigations of antimicrobial substances.\(^9\)

Calcium hydroxide introduced by Hermann in 1920, is the gold standard, against which the efficacy of all other medicaments has been tested since many years. Though there is significant literature about the use of Calcium hydroxide and its effects, the need to find other alternatives is evident through the failure of the calcium hydroxide to sterilize organisms within the dentinal tubules in many cases.\(^10\) The failure of Calcium hydroxide against various intracanal microorganisms, researchers then turned their attention to antibiotics and natural alternatives.

The use of antibiotics as intracanal medicaments began as early as 1951 where Grossman used the polyantibiotic paste for the first time known as PBSC consisting of Penicillin, Bacitracin, Streptomycin and Caprylate Sodium.\(^11\) Later, other broad spectrum antibiotics were introduced singly or in combination to overcome the polymicrobial endodontic infections of pulpal and periradicular tissues. Ledermix paste was then introduced by Schroeder and Tridian in 1960 which was a combination of a Tetracycline based antibiotic – Demeclocycline hydrochloride and a Corticosteroid component – Triamcinolone acetonide.\(^12\) The most recent addition of
this list of intracanal medicaments is the introduction of Triple Antibiotic Paste by Sato et al in 1992 consisting of Ciprofloxacin, Metronidazole and Minocycline.\textsuperscript{13}

This Triple Antibiotic Paste (TAP) became the mainstay of all intracanal medicaments as compared to the gold standard; Calcium hydroxide due to its bactericidal action and excellent antimicrobial activity. It is extensively being used in the Regenerative Endodontics as an intracanal medicament to disinfect the canal, prior to inducing blood clot formation. However, researchers have tried to change this combination ever since with various antibiotic and vehicles combinations.

Then through numerous \textit{in vitro} studies, efficacy of Double Antibiotic Paste (DAP) was proved to be equally effective as TAP. A randomized, double blinded study registered at Clinical Trials registry National Institute of Health(NIH-USA) Identifier:NCT00881491 is evaluating the treatment outcomes in permanent teeth with necrotic pulp and immature root development that undergo a regenerative procedure using a triple antibiotic paste (Ciprofloxacin, Metronidazole, Minocycline) versus a double antibiotic paste (Ciprofloxacin, Metronidazole) compared to the commonly used mineral trioxide aggregate (MTA) apexification treatment since 2009 and has been completed in February 2016.\textsuperscript{14}

Though this overcame the use of Minocycline which has a detrimental effect of discolouring teeth\textsuperscript{15} but the use of antibiotics always had an inherent risk of development of antibiotic resistance. So, the use of Biocides and herbal alternatives began; as intracanal medicaments from these sources do not develop resistance.\textsuperscript{16, 17} Also, the role of vehicles needs to be explored and used to its optimum benefit to have more effective, biocompatible and economical intracanal medicaments for thorough disinfection of root canals and thus, improving the success of pulp therapy and preserving the deciduous teeth.
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Literature related to the efficacy of intracanal medicaments in deciduous teeth is scanty as compared to many studies in the permanent teeth. Hence, an impending need is upon the pedodontic speciality to find a reasonable alternative, which can best adapt to primary tooth physiology with maximum clinical benefit and minimum side effects. Hence, in this study an attempt has been made to evaluate the antimicrobial activity of innovative combinations of intracanal medicaments and vehicles on selected bacteria isolated from deciduous molars with necrotic pulp.

“It is common sense to take a method and try it. If it fails, admit it frankly and try another. But above all, try something”

Franklin D. Roosevelt
REVIEW OF LITERATURE

1.2 LITERATURE REVIEW:

I. Pulp Therapy in Deciduous Teeth and it’s Importance:

II. Role of Intracanal medicaments:

III. Chlorhexidine as an intracanal medicament:

IV. Emergence of Triple Antibiotic Paste(TAP) as an intracanal medicament:

1. Antibiotics as intracanal medicament;
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   b. *In vitro* experiments to study effectiveness of mixed antibiotics;
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2. Concerns regarding use of Triple Antibiotic Paste(TAP);


4. Conclusion.

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1. Review of articles related to detection of endodontic microorganisms by culture;

2. Review of articles related to detection of endodontic microorganisms by Polymerase Chain Reaction.

3. Conclusion
I) Pulp Therapy in Deciduous Teeth and it’s Importance:

Various studies have stressed the importance of Deciduous teeth and the need for maintaining the deciduous teeth rather than extracting it. Primary teeth with pulpal involvement and excessive external or internal root resorption, or extensive bone loss, render the prognosis less favourable and are contraindicated for pulp therapy.\textsuperscript{18} Camp JH (1984), highlighted the fact that in young children, early removal of primary teeth is not recommended due to changes in the arch length, resulting in a mesial drift of the permanent teeth and consequent malocclusion. Some authors advocate the extraction of teeth with poor prognosis and placement of space maintainers. Yet, these appliances have some disadvantages functionally and in oral hygiene care.\textsuperscript{4} Holan G et al (1993) stated that preservation of the primary tooth is the best space maintainer for its successor, if pathological process can be resolved.\textsuperscript{2} Rocha CT et al (2008) have mentioned that deciduous teeth and the permanent successors are interdependent units, where both interact and depend on each other. Pulpal infection of a deciduous tooth can spread over the periradicular tissues causing alterations in the dental germ of the permanent successor if no treatment is done, i.e. pulp therapy or extraction. Cases of permanent teeth that show alteration in eruption or in development, as a consequence of infection of the preceding primary teeth, such as: hypoplasia, morphological alteration of the dental crown or total arrest of root formation have been reported in literature.\textsuperscript{5}

Pulp therapy is the ideal treatment modality inorder to save the deciduous tooth. To achieve this, many treatment procedures have been proposed such as indirect or direct pulp capping, partial pulpotomy, pulpotomy, and pulpectomy. Pulp therapy is ideal but expensive, takes minimum 2-3 visits and also co-operation of young children matters.
II) Role of Intracanal medicaments:

Many studies have explored the role of intracanal medicaments in the success of endodontic therapy. Martin H (1991) highlighted the role of disinfection as an crucial step to overcome the bacteria causing infection in the root canals and the dentinal tubules. Also, the review emphasized the use of bactericidal medicaments and their topical application to be more effective than antibiotic applications. Eradication of bacteria and necrotic debris from the canal by chemomechanical means of biomechanical preparation, thorough irrigation, and antibacterial medicaments enhance the successful outcome of endodontic therapy. Chong BS et al (1992) reviewed the role of intracanal medication. The authors considered the role to be secondary to biomechanical preparation and of more significance in infected root canals for controlled asepsis. They also insisted on bacteriological sampling to choose intracanal medicament for teeth which do not respond to treatment.

Silveira AM et al (2007) evaluated the response of peri-radicular tissues of infected canals to different intracanal medicaments during single visit or two visit procedures endodontic therapy in dogs. The animals were sacrificed after 6 months for histopathological analysis and it revealed that two visit endodontically treated teeth with intracanal medicaments showed higher success rate. Athanassiadis B et al (2007) in their review stated the fact that after thorough cleaning and shaping, maintaining the root canals in an disinfected state is of utmost importance and the intra canal medicament to be chosen depends on accurate diagnosis and the microorganisms inhabiting the root canals. They concluded that further research is required on the different intracanal medicaments available like calcium hydroxide, antibiotics, non-phenolic biocides, phenolic biocides and iodine compounds; to find the most suitable intracanal medicament. Kawashima N et al (2009) in their review
have concluded that use of intracanal medicaments should be performed more in multiple-visit endodontics especially calcium hydroxide due to its inherent advantages like inducing hard tissue formation.\textsuperscript{23} \textbf{Gondim JO et al (2012)} in their study using quantitative real-time polymerase chain reaction (qRT-PCR) have observed that though there was no statistically significant difference between the two intracanal medicaments i.e. calcium hydroxide with polyethylene glycol and calcium hydroxide with 2\% Chlorhexidine gel but there was significant difference between reduction in \textit{Porphyromonas gingivalis} (\textit{P. gingivalis}) after; biomechanical preparation only and placement of intracanal medicament (p<0.008).\textsuperscript{24}

III) Chlorhexidine as an intracanal medicament:

Various studies have been carried out using Chlorhexidine gel as an intracanal medicament. \textbf{McDonnell G et al (1999)} reviewed different antiseptics, of which Chlorhexidine a biocide; is an effective broad-spectrum antimicrobial drug due to it’s unique properties of substantivity and safety.\textsuperscript{25} \textbf{Oncaag O et al (2006)} evaluated the efficacy of 1\% Chlorhexidine gel against \textit{Enterococcus faecalis} in necrotic primary teeth \textit{in vivo}. They found that 1\% Chlorhexidine gel either alone or in combination with calcium hydroxide was more effective in reduction of \textit{E. faecalis} as compared to calcium hydroxide alone.\textsuperscript{26}

\textbf{Gomes BP et al (2006)} carried out an \textit{in vitro} study to determine the antibacterial activity of calcium hydroxide, 2\% chlorhexidine gluconate gel and a combination of both, by means of agar diffusion and direct contact test. They observed that 2\% chlorhexidine gluconate gel alone had the maximum antibacterial activity, followed by the combination and calcium hydroxide with sterile water had minimum activity.\textsuperscript{27} \textbf{Kanisavaran ZM (2008)}\textsuperscript{28} and \textbf{Mohammadi Z et al (2009)}\textsuperscript{29} individually carried out reviews on Chlorhexidine gluconate in endodontics and
further stressed the importance of disinfection of root canals. They also highlighted the advantageous properties of Chlorhexidine namely being a cationic molecule and hence, attaching to cell surfaces and possessing the unique property of substantivity.

**Vargas-Ferreira F (2010)** compared the antibacterial activity of an iodoform-based paste and a modified paste by the addition of a 2% Chlorhexidine gluconate gel against common endodontic pathogens in deciduous teeth and found them to be similar.\(^{30}\) **Ramani N et al (2012)** assessed antibacterial activity of ethanolic extract of propolis and chlorhexidine as intracanal medicaments against *Candida albicans* American Type Culture Collection (ATCC) 10231 (*C. albicans*) and *Enterococcus faecalis* ATCC 51299 (*E. faecalis*). They observed ethanolic extract of propolis to be more effective against *E. faecalis* while Chlorhexidine to be more effective against *C. albicans*.\(^{31}\)

**Anjaneyulu K et al (2014)** carried out a systemic review. Some studies found that calcium hydroxide when used alone is ineffective in eliminating post-operative pain but its efficacy can be improved by combination with other intracanal medicaments like Chlorhexidine and camphorated monochloro phenol.\(^{32}\) **Bhandari S et al (2014)** evaluated the antibacterial activity of 2% Chlorhexidine gel, Propolis and Calcium hydroxide against *Enterococcus faecalis* in an *in vitro* study and found out 2% Chlorhexidine gel to be the most effective intracanal medicament of the above three.\(^{33}\)

**Zare Jahromi M et al (2015)** studied the shear bond strength of composite restoration to dentin after application of Chlorhexidine and Triple antibiotic paste as medicament and concluded that though both medicaments did not interfere with the bond strength. Chlorhexidine increased the average bond strength due to its dentinal absorption and consequent absorption of the dentin bonding agents too.\(^{34}\)
IV) Emergence of Triple Antibiotic Paste (TAP) as an intracanal medicament:

1. Antibiotics as intracanal medicament:

   The local application of antibiotics has many advantages over the use of systemic antibiotics due to which antibiotics were begun to be used as intracanal medicaments. Numerous studies have been conducted using various combinations or individual antibiotics as *in vitro* and *in vivo* studies.

a. Rationale for local application of antibiotics as intracanal medicaments:

Abbott PV et al (1990) reviewed the role of antibiotics in endodontics as prophylactic and local or intracanal. They also advised the use of systemic administration of antibiotics only when indicated i.e., fascial space infections and fever or malaise and restrict the use of broad spectrum antibiotics for serious ailments in order to reduce the indiscriminate use of antibiotics.\(^{35}\) Mohammadi Z et al (2009) reviewed the use of antibiotics in endodontics and dental traumatology; few of them were Tetracyclines, corticosteroids like Ledermix and another mixture of antibiotics consisting of Clindamycin, Ciprofloxacin, Minocycline and Metronidazole as commercially available agents may not be ideal.\(^{36}\) Mohammadi Z (2009) individually researched the use of antibiotics as intracanal medicaments and concluded that they have advantage over systemic use as it reduces the adverse systemic effects associated, and is also effective in necrotic pulpless teeth and periapical tissues.\(^{37,38}\)

Mohammadi Z (2009) again solely reviewed the use of Tetracyclines in endodontics and dental trauma especially root resorption; and highlighted the antibacterial substantivity of tetracycline which is upto 12 weeks, a Mixture of a Tetracycline isomer, an Acid, and a Detergent (MTAD) which is upto 4 weeks and another preparation referred to as Tetraclean which is a mixture of Doxycycline, acid and a
detergent. Kayalvizhi G et al (2013) conducted a review of the topical application of antibiotics referred to as Lesion Sterilization and Tissue Repair therapy in primary teeth. They concluded that more research with better study designs, long follow-ups and radiographic and histological evaluations were needed.

b. In vitro experiments to study effectiveness of mixed antibiotics:

Out of the many in vitro studies, Ingham HR et al (1975) found Metronidazole to be highly effective against anaerobes through a literature review and found it having antibacterial activity in anaerobic infections like ulcerative gingivitis. In another study by Hoshino E et al (1988), Metronidazole was indeed bactericidal in action and killed 99% of the bacteria grown from carious lesions on Brain Heart Infusion-Blood agar and also on carious lesions on freshly extracted teeth.

Sato T et al (1992) carried out in vitro and in situ studies using a mixture of Ciprofloxacin, Minocycline and Metronidazole with and without Rifampicin against oral bacteria from carious lesions, root canals, periodontal pockets and also plaque on deciduous teeth. After application of the drug mixture, bacteria could not be recovered, thus proving its efficacy in sterilization of infected dentin. Sato T et al (1993) in an in vitro susceptibility of bacteria from carious and endodontic infections of human deciduous to mixed antibacterial drugs consisting of Ciprofloxacin, Metronidazole, plus a third antibiotic: Amoxicillin, Cefaclor, Cefroxadine, Fosfomycin or Rokitamycin. All the drug mixtures were bactericidal and thus effective in sterilizing the infections in situ. Sato I et al (1996) conducted a study to check the efficacy of a mixture of Ciprofloxacin, Metronidazole and Minocycline in situ against microorganisms residing in the deeper layers of root dentin and found it to be bactericidal. Hoshino E et al (1996) carried an in vitro study to ensure
susceptibility of bacteria from infected root dentin to a mixture of Ciprofloxacin, Minocycline and Metronidazole with and without Rifampicin. The results confirmed the bactericidal effect of the employed drug mixture.\(^{45}\)

**Shabahang S et al (2003)** investigated the antibacterial effect of MTAD (a mixture of a tetracycline isomer, an acid, and a detergent) with that of NaOCl (Sodium hypochlorite) with and without EDTA (Ethylene diamine tetraacetic acid) against *Enterococcus faecalis* and found MTAD to be an effective antibacterial medicament.\(^{46}\) **Adl L et al (2012)** compared the antibacterial activity of the Triple Antibiotic mixture with saline, and 2% Chlorhexidine respectively and Minocycline with saline, and for Calcium hydroxide with saline, and 2% Chlorhexidine respectively by agar well diffusion assay. Triple Antibiotic mixture had the maximum antibacterial activity against *Enterococcus faecalis*.\(^{47}\) **Kuşgöz A et al (2013)** conducted an *in vitro* study of Calcium hydroxide with different proportions of TAP against *Enterococcus faecalis* and found TAP to be most effective as compared to all combinations of Calcium hydroxide with TAP.\(^{48}\)

c. **In vivo studies reported in primary teeth:**

**Kargül B et al (2001)** investigated the antibacterial activity of a short-term topical application of ornidazole against anaerobic microorganisms and found 94.53% reduction in the microorganisms.\(^{49}\) **Ehrmann EH et al (2003 and 2007)** compared the efficacy of Ledermix paste, calcium hydroxide paste and no dressing following root canal instrumentation in reduction of post-operative pain and found Ledermix to be the most effective of all medicaments, though the difference was not statistically significant in the latter study.\(^{50,51}\)

**Takushige T et al (2004)** carried out an *in vivo* study following the the series of *in vitro* studies to evaluate the clinical success of application of TAP in endodontic
treatment of deciduous teeth. At the Cariology Research Unit of the Niagata University School of Dentistry, they brought the concept of ‘Lesion Sterilization and Tissue Repair (LSTR)’ using an antibacterial drug mixture to disinfect the carious lesions and thus, cause the healing of damaged tissues. They placed a mixture of Ciprofloxacin, Metronidazole and Minocycline (3Mix) in a ratio of 1:3:3 with combination of Macrogol and Propylene glycol(MP) to form a Triple Antibiotic Paste(TAP); in a specially prepared medication cavity at the orifices of the root canals.

**Fig. 1:** The following is the schematic representation of LSTR 3Mix-MP Non-Instrumentation Endodontic Technique(NIET) for infected deciduous teeth (Figure 1).

![Schematic representation of LSTR 3Mix-MP Non-Instrumentation Endodontic Technique](image)


The authors also included teeth with physiologic resorption and the treatment consisted of placement of 3Mix-MP on the pulpal floor, later sealed by glass-ionomer cement and further placement of direct resin bonded inlay; was completed in a single visit. Later metal inlays were luted with resin cement to restore the tooth in a separate visit, thus requiring a maximum of two visits to the dentist. Short-term evaluation was
done within ten days to a month, and teeth were regarded as clinically successful if they were asymptomatic. Long-term evaluation was done at routine visits or as recall and normal exfoliation of the deciduous teeth following treatment was followed up. They concluded that with a certain precautions, LSTR-therapy employing 3Mix-MP provided an excellent outcome in endodontically infected deciduous teeth.\textsuperscript{52}

Takushige T et al explored other applications of this 3Mix-MP like in non-surgical treatment of carious lesions diagnosed as pulpitis and retreatment of failed endodontic treatments. \textbf{Takushige T et al (2008)} explored the possibility of non-surgical treatment of teeth diagnosed with Pulpitis either Reversible or Irreversible, treated locally with TAP without a pulpectomy procedure. Treatment consisted of application of TAP on the pulpal floor of the carious lesion leaving the infected dentin intentionally behind, followed by sealing with glass-ionomer cement and lastly restored by resin inlays. 95\% of the cases were considered clinically successful and remineralization of softened dentin was observed on postoperative radiographs in cases where softened dentin was left behind. Thus, the authors concluded that 3Mix-MP could be further researched by conducting randomized clinical trials for it’s application in treatment of Reversible or Irreversible Pulpitis.\textsuperscript{53}

\textbf{Prabhakar AR et al (2008)} evaluated clinical and radiographic success of treatment of infected deciduous teeth using TAP in a randomized clinical trial. Out of the two groups, one group was treated with TAP following pulpotomy while in the other group following partial pulpectomy and then sealed with glass ionomer followed by restoration with resin composite. Clinical evaluation was done within a month. Both clinical and radiographic evaluation was done every six months for a period of one year, to find that both techniques were successful but application of TAP following partial pulpectomy had better success.\textsuperscript{54} \textbf{Takushige T et al (2009)}
further explored the application of TAP in re-treatment endodontics without removal of the previous defective root canal obturation. All previously obturated teeth with periapical radiolucency visible on radiographs were treated by placement of TAP on the obturated root canals at the orifices of root canals. Clinical success was evaluated on the basis of absence of any signs and symptoms as also reduction in periapical radiolucency and alveolar bone resorption. The authors concluded that this technique had a good clinical outcome and needs well designed clinical trials to establish TAP as an treatment modality.\textsuperscript{55}

\textbf{Nakornchai S et al (2010)} carried out a single-blinded randomized clinical trial with pulpally involved deciduous molars with poor prognosis. The two interventional groups consisted of 3Mix or Vitapex followed by immediate placement of preformed stainless steel crowns. After the treatment, clinical evaluation was done at one week postoperative, 6 months and 12 months whereas, radiographic follow-up was done at 6 months and 12 months postoperatively. At 6 months, both 3Mix and Vitapex treated groups revealed clinical success and at 12 months both groups presented with a case of gingival abscess each. Radiographic success in both groups at 6 months was similar with two teeth showing internal resorption in 3 Mix treated group and one more tooth at 12 months. There was no statistically significant difference amongst both groups clinically and radiographically.\textsuperscript{56}

\textbf{Agarwal M et al (2011)} conducted a study to evaluate the clinical efficacy of two different non-instrumentation endodontic techniques i.e., LSTR and Pulpotec in comparison to conventional Zinc Oxide Eugenol (ZOE) pulpectomy technique at 1, 3, 6 and 12 months. Additional radiographic evaluation was done at 3,6 and 12 months and the authors concluded that both LSTR and Pulpotec pulpotomies could be considered viable alternatives to conventional ZOE pulpectomies but studies with
longer follow-ups were needed.\textsuperscript{57} Taneja S et al (2012) used the TAP for the non-surgical treatment of a chronic periapical lesion. When an intracanal dressing of calcium hydroxide failed to relieve the symptoms, TAP was used instead for a period of 3 months and the tooth was clinically asymptomatic and also showed reduction in the periapical radiolucency, radiographically.\textsuperscript{58}

\textbf{Trairatvorakul C et al (2012)} did a long follow-up study of 24-27 months using TAP for non-instrumentation endodontic treatment of deciduous mandibular molars. Contrary to the prior studies, long term radiographic success was low as compared to the clinical success of this treatment modality. Hence, the authors concluded that it cannot replace the conventional pulpectomy in long term.\textsuperscript{59}

\textbf{Gomes-Filho JE et al (2012)} evaluated the response of rat subcutaneous tissue to TAP or calcium hydroxide filled polyethylene tubes. Later at an interval of 7, 15, 30, 60, and 90 days tissues surrounding the tubes were incised and histologically analyzed for tissue response. The tissue response was similar in all medicaments as compared to the placebo control and reduced in severity from 30 days \textit{in vivo}. Also, the carriers or vehicles did not interfere with the reaction of the medicaments.\textsuperscript{60}

\textbf{Tulsani SG et al (2014)} evaluated the antibacterial efficacy of 2.5\% Sodium hypochlorite(NaOCl) and BioPure MTAD against \textit{Enterococcus faecalis} in deciduous teeth using Real Time Polymerase Chain Reaction. Both irrigants were clinically effective in reducing \textit{Enterococcus faecalis} in comparison to saline but further studies are needed to recommend the use of MTAD in deciduous teeth.\textsuperscript{61}

\textbf{Saleh A et al (2014)} have discussed the problem of treating endo-perio lesions, which show delayed radiographic healing due to the presence of microorganisms. This group of authors have used triple antibiotic paste (TAP) alongwith root-canal sealer for improved healing and their study recommends the use
of triple antibiotic paste (TAP) along with periodontal treatment in management of endo-perio lesions to improve success rate and periodontal health.\textsuperscript{62} Pai S et al (2014) tested the effect of calcium hydroxide and triple antibiotic paste as intracanal medicaments on the incidence of inter-appointment flare-up in diabetic patients. Calcium hydroxide had a flare-up in 16\% of the cases and triple antibiotic paste had none; in comparison to no intracanal medicament which had a flare-up in 50\% of the cases. Hence, both calcium hydroxide and TAP were effective for managing inter-appointment flare-ups in diabetic patients. Triple antibiotic paste was the most effective intracanal medicament in preventing the occurrence of flare-up in diabetic patients.\textsuperscript{63}

Burrus D et al (2014) reviewed and provided the results of three clinical case applications of TAP in the vehicle, propylene glycol. The purpose of this short literature review was to find current clinical evidence supporting the use of LSTR non-instrumentation endodontic technique in clinical practice as an alternative to pulpectomies and extraction of non-vital deciduous teeth.\textsuperscript{64}

Johns D et al (2014) evaluated the healing after 18 months following nonsurgical treatment of periapical lesions using Photo Activated Disinfection (PAD), triple antibiotic paste (TAP) and Calcium hydroxide as intracanal disinfectant. Twenty patients in each of the groups with periapical lesions in the maxillary and mandibular anterior region were treated. Failure was seen in 15\% of cases in calcium hydroxide group, 5\% in triple antibiotic paste, whereas PAD group had no failure. PAD and TAP were equally effective as an intracanal disinfectant whereas calcium hydroxide was significantly less effective.\textsuperscript{65}
2. Concerns regarding use of Triple Antibiotic Paste (TAP):

There were a few concerns with this technique as were its advantages! Pallasch TJ (2000) highlighted the main concerns like the development of antibiotic resistance and superinfection from pre-existing resistant microbes. The antibacterial drugs might promote bacterial chromosomal mutations, gene transfer from resistant microbes to non-resistant microbes and latent gene expression might be other concerns associated with antibiotic usage. Ferreira MB (2010) evaluated the cytotoxicity of different antibiotics used in endodontic procedures as intracanal medicaments namely Ciprofloxacin, Clindamycin and Metronidazole. They studied different concentrations and time related cytotoxicity of all these antibiotics and found that all antibiotics were cytotoxic to human gingival fibroblasts at higher concentrations and concentrations of 5 and 50 mg/L produced viable fibroblasts at all experimental times. Also, cell viability after application of all antibiotics for 24 hrs was maximum and Metronidazole was the least cytotoxic while Ciprofloxacin was the most cytotoxic in the time-dependent analysis.

Kim JH et al (2010) reported a case of an attempt at revascularization of necrotic immature permanent anterior tooth by placement of TAP for six weeks leading to dark discoloration. Hence, they carried out an in vitro experiment with extracted permanent teeth with individual antibiotics as intracanal medicaments and concluded that only Minocycline caused discoloration and use of dentin bonding agents reduced but could not eliminate the occurrence of discoloration.

Ruparel NB et al (2010) studied the effect of the commonly used intracanal medicaments for the regenerative endodontic procedures of immature necrotic permanent teeth. They studied the effect of TAP, modified TAP (Ciprofloxacin, Metronidazole and Cefaclor), Double Antibiotic Paste (DAP) consisting of
Ciprofloxacin and Metronidazole; and lastly Augmentin only in comparison to Calcium hydroxide and its effect on Stem Cells of Apical Papilla(SCAP) which are important for regeneration and apexogenesis of the immature necrotic tooth. They wanted to observe the concentration dependant toxicity of all the intracanal medicaments on SCAP, and concluded that all four antibiotic based intracanal medicaments had a detrimental effect on SCAP survival whereas Calcium hydroxide promoted the SCAP survival and proliferation.⁶⁹

**Turk T et al (2015)** studied the time-dependent effect of intracanal medicaments used for regenerative procedures on the dislocation resistance of Mineral Trioxide Aggregate (MTA). After placement of intracanal medicaments for different time periods, dislocation resistance was measured by a push out test for MTA placed in the coronal third of the root canal. The researchers observed that the antibiotics significantly reduced the dislocation resistance of MTA after 12 weeks as compared to one week and steep decrease in the dislocation resistance was seen with TAP and DAP as early as two weeks while, on the contrary calcium hydroxide had no effect on dislocation resistance as long as two-four weeks. Hence, the authors arrived at a clinical decision of limiting the time period of application of TAP and DAP for regenerative endodontics for a maximum period of two weeks whereas calcium hydroxide could be kept in the root canals for a period of four weeks.⁷⁰

**Yilmaz S et al (2016)** also had similar observations that application of TAP and DAP for endodontic regenerative procedures reduced the microhardness of human dentin discs in vitro after four weeks. They also compared the reduction in the Knoop hardness number of the dentin discs with calcium hydroxide and control consisting of no medicament and they had similar findings that calcium hydroxide did not reduce the microhardness as the control. Hence, we can conclude that application
of either TAP or DAP should be with caution as it reduces the microhardness of the treated immature necrotic tooth and thus makes it weaker.\textsuperscript{71}

3. \textbf{Modifications of Triple Antibiotic Paste (TAP):}

To overcome the disadvantages associated with the use of TAP, various authors came up with their innovative modifications, so that this intracanal medicament, which has potent antibacterial activity could be utilized in demanding clinical situations. Pinky C \textit{et al} (2011) conducted an \textit{in vivo} using two different combinations of antibiotics in deciduous teeth with a clinical and radiographic evaluation at a period of 3, 6, and 12 months. The two combinations consisted of Ciprofloxacin, Minocycline and Metronidazole i.e. TAP and the modified combination consisted of Ciprofloxacin, Minocycline and Ornidazole. Both the antibiotic mixtures were mixed with propylene glycol. The principal investigator followed the technique by Takushige \textit{et al} of preparing a medication cavity for placement of the antibiotic pastes followed by glass ionomer restoration after resolution of signs and symptoms at 15 day recall. Stainless steel crown restorations were placed at the 30 days recall. Both clinical and radiographic evaluation was done at the recall visits to evaluate the success of the therapy performed using the two combinations of antibiotic drugs. The authors concluded that both the antibiotic combinations had similar clinical and radiographic success but needed clinical and histological follow-up till exfoliation of the treated deciduous teeth.\textsuperscript{72}

\textbf{Sabrah AH \textit{et al} (2013)} executed an \textit{in vitro} study comparing the antibacterial activity of TAP and DAP against \textit{Enterococcus faecalis} and \textit{Porphyromonas gingivalis} in comparison to calcium hydroxide. Both TAP and DAP were significantly more efficient in reduction of bacterial biofilms in comparison to calcium hydroxide. Hence, the investigators concluded that DAP is comparable to TAP in its antibacterial
activity and can substitute TAP. Rishi Nanda et al (2014) used a similar study design and methodology to conduct a randomized clinical trial using the same combination of drugs as Pinky et al. They concluded that both the antibiotic mixtures had similar efficacy and these both antibiotic pastes could be considered viable alternative to conventional pulpectomy but more clinical trials were needed to establish this novel therapy.

Sabrah AH et al (2015) carried out an in vitro study to determine effect of different concentrations of TAP and DAP on human Dental Pulp Stem Cells (DPSCs) and established Enterococcus faecalis biofilm. The authors concluded that all the concentrations had significant antibacterial activity against the established Enterococcus faecalis biofilm. Also, DAP was non-toxic to DPSCs at higher concentration upto 0.5 mg/ml whereas TAP at concentrations except 0.125 mg/ml significantly reduced the viability of DPSC. Hence, inspite of similar antibacterial activity DAP has advantage of being more biocompatible with DPSC.

Albuquerque MT et al (2015) used TAP-mimic scaffolds to eliminate established Actinomyces naeslundii dentin biofilm. A burst release of all the three antibiotics in the TAP was observed in the first 24 hours followed by sustained release for 4 weeks of Ciprofloxacin and Metronidazole only. The TAP-mimic scaffolds have a promising application in the emerging field of regenerative endodontics.

Sabrah AH et al (2015) also investigated the residual antibacterial effect of TAP and DAP during treatment of human radicular dentin using different concentrations of the two antibiotic pastes. Both the antibiotic pastes had significant residual antibacterial effect. DAP showed longer residual antibacterial effect as compared to the same concentration of TAP. Algarni AA et al (2015) studied the inhibitory effect of gels having concentrations of 1mg/ml Modified Triple Antibiotic
Paste (MTAP-Ciprofloxacin, Metronidazole and Clindamycin) and Double Antibiotic Paste (DAP- Ciprofloxacin with Metronidazole) against Enterococcus faecalis and Porphyromonas gingivalis biofilms. No significant differences in biofilm-inhibitory effects between MTAP and DAP gels were observed.⁷⁸

Kim KW et al (2015) explored the effects on the attachment and proliferation of dental pulp stem cells (DPSCs) of dentin treated with two different concentrations of Double Antibiotic Paste (DAP) and Ethylene diamine tetraacetic acid (EDTA). Dentin treated with concentration of 1 and 500 mg/ml of DAP and EDTA thereafter, caused significant increase in DPSCs attachment in comparison to the dentin treated with the DAP alone. The 500 mg/ml concentration of DAP with or without EDTA caused significant reductions in DPSCs proliferation. However, the treatment of dentin with 1 mg/ml of DAP did not have significant negative effects on DPSCs proliferation regardless of the use of EDTA.⁷⁹ Park HB et al (2015) reported a case using amoxicillin-containing TAP for revascularization of necrotic immature teeth to prevent tooth discoloration. At the 1 yr follow up, the teeth were asymptomatic on clinical examination. Radiographic examination revealed complete resolution of the periapical radiolucency, and closed apex with obvious periodontal ligament space.⁸⁰

4. Conclusion:

Hence, it again highlights the antibacterial activity of DAP being equivalent to TAP and various Modifications of TAP, and the subsequent application of DAP can be considered the standard regimen for regenerative endodontics.

V) Role of vehicles/carrier for dispensing the intracanal medicaments:

There are a few studies regarding the role of vehicles in comparison to the research conducted on intracanal medicaments. Vehicles play an important role as carriers for the medicaments or as dispensing medium and many vehicles have been
utilized till date. **Cruz EV et al (2002)** investigated the penetration effect of propylene glycol into root dentin. Propylene glycol delivered dye through the root canal system rapidly and more effectively indicating its potential use in delivering intracanal medicaments.  

**Gomes BP et al (2002)** carried out an *in vitro* study of calcium hydroxide with different vehicles against the common endodontic pathogens. They tested the antimicrobial activity of the calcium hydroxide combination with Camphorated Monochlorophenol (CMCP), glycerine, anesthetic, saline, water and polyethylene glycol (PEG) by agar diffusion method. The intracanal medicaments with vehicles were filled inside stainless steel cylinders and placed on cultured agar media. After incubation, antimicrobial activity was recorded by measuring the zones of inhibition. The test medicaments with the vehicle combination in increasing order of their antimicrobial activity were Calcium hydroxide + PEG (least activity), Calcium hydroxide + water, Calcium hydroxide + saline, Calcium hydroxide + anesthetic, Calcium hydroxide + glycerine and Calcium hydroxide + CMCP + glycerine. The authors concluded that oily followed by aqueous or viscous vehicles enhanced antimicrobial activity and diffusion of calcium hydroxide(CH) and hence, the type of vehicle played a crucial role in dispensing an intracanal medicament.  

**Oliveira JC et al (2010)** studied the effect of necrotic tissue and serum on the antimicrobial activity of endodontic medicaments like calcium hydroxide/glycerin paste, calcium hydroxide/chlorhexidine paste, calcium hydroxide/camphorated paramonochlorophenol/glycerine paste, and chlorhexidine/zinc oxide paste. They found that the presence of necrotic tissue and serum had a detrimental effect on the medicament activity. While Calcium hydroxide pastes in glycerin or Chlorhexidine were the most affected endodontic medicaments; calcium hydroxide / camphorated paramonochlorophenol / glycerin paste were the least affected. **Silveira CF et al**
(2011) evaluated the antimicrobial activity of Calcium hydroxide in four different combinations with 2% Chlorhexidine gel, CMCP and propylene glycol (PG), PG only and saline against Enterococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa and Streptococcus mutans. The time period for the four combination of pastes to kill the microorganisms was recorded using broth dilution method and E. faecalis was found to be the most resistant organism. Calcium hydroxide with CMCP and PG and propylene glycol alone were the most effective vehicles to kill Enterococcus faecalis, and Staphylococcus aureus in shortest time.  

Parasuraman VR et al (2012) reviewed that the ideal vehicle for dispensing antibiotics in root canal should facilitate better diffusion of medicament through dentinal tubules and anatomical aberrations like fins, isthmuses and blocked canals. Therefore diffusion of antibiotic into cementum and periradicular tissue may be advantageous and specifically enhanced by the use of vehicles, as also the vehicles may have synergistic antimicrobial activity with the endodontic medicaments.

Ganesh MR et al (2013) evaluated the effect of three different vehicles namely saline, CMCP+glycerine and glycerine only on the activity of calcium hydroxide against Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, and Streptococcus sanguis. Their results showed that although all the medicament-vehicle combinations exhibited antimicrobial activity, Calcium hydroxide with CMCP+glycerine, an oily vehicle combination; exhibited the maximum effect which was similar to the results by Gomes BP et al.  

Ganesh M et al (2014) evaluated the antibacterial effect of calcium hydroxide (CH) in different vehicles in an in vitro model. Calcium hydroxide paste prepared with two conventionally used vehicles namely, camphorated monochlophenol, distilled water and propylene glycol. The antibacterial activity of these paste were tested against five
Review of Literature

microorganisms that can commonly occur in the infected root canals namely Hemophilus streptococcus, Enterococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans. The results of this study indicate that a paste of CH made with propylene glycol exerts significant antibacterial action. Hence, it can be recommended for use as an intracanal medicament in preference to a paste prepared with a tissue toxic phenolic compound like camphorated monochlorophenol.\textsuperscript{87}

Kim D et al (2014) reviewed the antimicrobial effect of Calcium hydroxide is related to the hydroxyl ions released in an aqueous environment, which affects cytoplasmic membranes, proteins, and the DNA of microorganisms. Calcium hydroxide has a wide range of antimicrobial effects against common endodontic pathogens, but it is less effective against specific species such as E. faecalis or C. albicans. The addition of vehicles or other agents might contribute to the antimicrobial effect of Calcium hydroxide.\textsuperscript{88}

Shaik J et al (2014) analyzed the sustained release of intracanal medicaments with or without a carrier and testing their antimicrobial efficacy in root canal against Candida albicans and Enterococcus faecalis. A total of 80 single rooted anterior teeth were selected, root canal preparation was done, and teeth were divided into two halves and contaminated with C. albicans and E. faecalis, which were further divided into four test groups each according to intracanal medicaments used. Chitosan was used as vehicle for triple antibiotic paste(TAP) and calcium hydroxide-Ca(OH)\textsubscript{2} and antimicrobial assessment was performed on second and seventh day. Combination of TAP + chitosan and Ca(OH)\textsubscript{2} + chitosan produced better results compared with the combination of medicaments with saline.\textsuperscript{89} Grover C et al (2014) evaluated the calcium ion release and change in pH on combining calcium hydroxide with different
vehicles. Some in vitro studies have shown that the type of vehicle has a direct relationship with the concentration and the velocity of ionic liberation as well as with the antibacterial action when the paste is carried into a contaminated area. Forty single rooted mandibular first premolar teeth were decoronated for this study. Working length was established and the root canals were enlarged and irrigation accomplished with 2 ml of NaOCl solution after every file. The teeth were then randomly divided into four groups. The canals were then packed with different preparations of calcium hydroxide using the following vehicles-distilled water, propylene glycol, gutta-percha points and chitosan. Calcium ion release in different groups was analyzed using an ultraviolet spectrophotometer. The change in pH of was determined using a pH meter. Propylene glycol showed the maximum cumulative drug release of 81.97% at the end of 15 days and showed the highest pH value (10.35) after 30 days. \textsuperscript{90} Nalawade TM et al (2015) evaluated the bactericidal activity of propylene glycol, glycerine, polyethylene glycol 400 (PEG 400), and polyethylene glycol 1000 (PEG 1000) against selected microorganisms by broth dilution method and then streaking out the results. Five vehicles, namely propylene glycol, glycerine, PEG 400, PEG 1000, and combination of propylene glycol with PEG 400, were tested for their bactericidal activity. The minimum bactericidal concentration was noted against four standard strains of organisms, i.e. \textit{Streptococcus mutans} American Type Culture Collection (ATCC) 25175, \textit{Staphylococcus aureus} ATCC 12598, \textit{Enterococcus faecalis} ATCC 35550, and \textit{Escherichia coli} ATCC 25922, using broth dilution assay. Propylene glycol was effective against three organisms namely \textit{S. mutans \textit{E. faecalis}} \textit{E. coli} and its bactericidal activity was at \textit{50\%, 25\%} and \textit{50\%} respectively. PEG 1000 was effective against \textit{S. mutans} and \textit{E. coli} at 25\%. Hence propylene glycol was effective on more number of organisms of which \textit{E. faecalis} is a known resistant species. PEG
1000 was bactericidal at a lower concentration but was effective on two organisms only.\textsuperscript{91}

\textbf{Afkhami F et al (2015)} investigated antibacterial characteristic and \textit{Enterococcus faecalis (E. faecalis)} biofilm suppression effect of different vehicles of calcium hydroxide as intracanal medicaments in short and long-term. Fifty-four human single-root teeth were contaminated with \textit{E. faecalis} bacteria. The teeth were randomly divided into three experimental (n = 16) and one control group (n = 6). Each group was then exposed to various intracanal medicaments, namely calcium hydroxide paste (group 1), calcium hydroxide with Chlorhexidine (group 2), calcium hydroxide with silver nanoparticles suspension (AgNPs) (group 3), and saline as the control group (group 4). Cultures were made from each group after one week and one month, and the number of colonies were counted. All the intracanal medicaments resulted in significant decrease in number of colonies compared to control group in both incubation periods. After one week, the mixture of calcium hydroxide and AgNPs was the most effective medicament against \textit{E. faecalis} bacteria (p \textless 0.05). No significant difference in antibacterial effect of the medicaments existed after one month incubation period (p > 0.05).\textsuperscript{92}

\textbf{Junior JS et al (2015)} reviewed that several substances have researched to act as vehicles associated with calcium hydroxide. The specific type of vehicle is directly related to the effectiveness of the ionic dissociation, antimicrobial action, and biocompatibility of this medication. Histological evaluation of the biocompatibility of calcium hydroxide associated with a new vehicle (triethanolamine), compared with polyethylene glycol, saline solution, and olive oil was done. Triethanolamine showed higher biocompatibility, especially as compared with the specimens from PEG and olive oil groups.\textsuperscript{93}
VI) Microbiology of Endodontic infections in necrotic deciduous teeth:

The initial studies to recover the bacteria associated with endodontic infections were thought to be aerobic or facultative anaerobic due to limitations in culture techniques. With the advancement of culture techniques, it was revealed that strict anaerobes dominated as the time elapsed and teeth became necrotic. Following is the review of literature pertaining to identification of endodontic bacteria in infected deciduous teeth by means of culture.

1. Review of articles related to detection of endodontic microorganisms by culture:

Hu Yw et al (1998) analyzed the microbial flora of infected human deciduous teeth root canals using immediate-enzyme method. The bacterial profile was mixed in nature both in acute and chronic infections, anaerobes were dominantly identified. B. gingivalis and F. nucleatum have been more frequently isolated from root canals with acute periapical inflammation than that with chronic ones (P<0.05). In chronic cases, Veillonella especially V. parvula, were more frequently isolated than acute inflammation.94 Pazelli LC et al (2003) evaluated the bacterial prevalence in root canals of human deciduous teeth with necrotic pulp and periapical lesions using bacterial culture. Anaerobic microorganisms were found in 96.7% of the samples, black-pigmented bacilli in 35.5%, aerobic microorganisms in 93.5%, Streptococci in 96.7%, and S. mutans in 48.4%. The authors concluded that in human deciduous teeth root canals with necrotic pulp and periapical lesions the infection is polymicrobial, with a large number of microorganisms and a predominance of Streptococci and anaerobic microorganisms.95
Brook I (2003) mentioned that organisms that predominate in pulpitis and dento-alveolar abscess are *Prevotella*, *Porphyromonas*, *Fusobacterium*, and *Peptostreptococcus* spp. Treatment of caries involves removal of all affected tooth structure and proper replacement with a restorative material. Once pulpitis has developed the infected tissue should be removed and root canal therapy instituted, or the tooth should be extracted. Extraction, root canal therapy and/or drainage of pus usually are indicated for an abscess. Antimicrobial therapy supplementing the dental care should be considered, especially when local or systemic spread of the infection is suspected. Penicillin or amoxicillin are generally effective against most of the aerobic and anaerobic bacteria recovered. The patient whose oral cavity may harbor penicillin-resistant organisms should be considered for treatment with drugs effective against these organisms. These agents include Amoxicillin-clavulanate, Clindamycin or the combination of Metronidazole plus Amoxicillin or a Macrolide.⁹⁶

Gomes BP et al (2004) investigated the root canal microbiota of primary and secondary root-infected canals and the association of constituent species with specific endodontic signs and symptoms. Their findings indicate potential complex interactions of species resulting in characteristic clinical pictures which cannot be achieved by individual species alone. They also indicate that the microbiota of primary infected canals with apical periodontitis differs in number and in species from the secondary infected canals by using the culture technique.⁹⁷ da Silva LA et al (2006) evaluated the bacterial profile in root canals of human primary teeth with necrotic pulp and periapical lesions using bacterial culture. A total of 20 primary teeth with necrotic pulp and radiographically visible radiolucent areas in the region of the bone furcation and/or the periapical region were selected. After crown access, 4 sterile absorbent paper points were introduced sequentially into the root canal for collection
of material. After 30 secs, the paper points were removed and placed in a test tube containing reduced transport fluid (RTF) and were sent for microbiological evaluation. Anaerobic microorganisms were found in 100% of the samples, black-pigmented bacilli in 30%, aerobic microorganisms in 60%, *Streptococci* in 85%, gram-negative aerobic rods in 15% and *Staphylococci* were not quantified. *Mutans streptococci* were found in 6 root canals (30%), 5 canals with *Streptococcus mutans* and 1 canal with *Streptococcus mutans* and *Streptococcus sobrinus*. It was concluded that in root canals of human primary teeth with necrotic pulp and periapical lesions, the infection is polymicrobial with predominance of anaerobic microorganisms.\(^98\)

**Rana V et al (2009)** reviewed that, vast majority of diseases of the dental pulp and the periradicular tissues are associated with microorganisms. A thorough understanding of these organisms and their relationship to clinical symptoms is necessary to formulate a sound approach to deciduous root canal therapy.\(^99\) **Ledezma-Rasillo G et al (2010)** identified cultivable microorganisms from primary teeth with necrotic pulps. This experimental study included 21 patients of both sexes between 4 and 7 years of age with necrotic pulps in primary teeth. Twenty-one maxillary and mandibular molars containing at least 1 necrotic canal, an abscess or sinus tract, one or more radiolucent areas in the furcation or periapical region, teeth having at least two thirds of root length, and carious lesions directly exposed to the oral environment were included. After antisepsis of the oral cavity, anesthesia of the affected tooth, and isolation and disinfection of the operative field, 3 sterile absorbent paper points were sequentially placed for 30 seconds for the collection of samples. The samples were immediately processed in an anaerobic chamber, and all isolated microorganisms were identified. Anaerobic species (anaerobic facultative and moderate anaerobes) were isolated in all root canals; 68.4% of root canal samples studied showed a
polymicrobial nature. Most of the isolate consisted of *Bifidobacterium spp* and *Streptococcus intermedius*. Other less frequently encountered species were *Actinomyces israelii, Bifidobacterium spp, Clostridium spp*, and *Candida albicans*. Results indicate the existence of combinations of bacterial species in root canal infections of the primary dentition with necrotic pulps, anaerobic bacteria predominating.\(^{100}\)

**Sato T et al (2012)** performed the profiling of the microbiota in infected root canals using anaerobic culture and molecular biological techniques for bacterial identification. The overwhelming majority of the isolates from infected root canals were found to be anaerobic bacteria, suggesting that the environment in root canals is anaerobic and therefore support the growth of anaerobes.\(^{101}\)

### 2. Review of articles related to detection of endodontic microorganisms in Deciduous teeth by Polymerase Chain Reaction (PCR).

With the advent of molecular techniques even difficult to grow bacteria were now identifiable! Literature related to identification of bacteria in endodontic infections of Deciduous teeth by means of PCR is scanty. The following are the studies using PCR for detection of endodontic pathogens from infected deciduous teeth.

**Cogulu D et al (2007)** investigated the presence of *Enterococcus faecalis* in endodontic infections in both deciduous and permanent teeth by culture and polymerase chain reaction (PCR) methods. *E. faecalis* was cultured from 8 (18%) of 45 necrotic deciduous teeth and from 10 (26%) of 38 necrotic permanent teeth. PCR detection identified the target species in 10 (22%) and 12 (32%) of necrotic deciduous and permanent teeth respectively. The results of the present study confirmed that both culture and PCR methods are sensitive to detect *E. faecalis* in root canals.\(^{102}\)**Cogulu**
D et al (2008) studied the presence of selected pathogens (Actinomyces israelii, Candida albicans, Enterococcus faecalis, Fusobacterium nucleatum, Porphyromonas endodontalis, Porphyromonas gingivalis, Prevotella intermedia, Streptococcus intermedius, Treponema denticola, Parvimonas micra, Tannerella forsythensis, Enterococcus faecium, Prevotella melaninogenica) in infected root canals using PCR.

T. denticola (p = 0.012, 0.02) and E. faecalis (p = 0.012, 0.04) were highly associated with periapical radiolucency and previous pain, while P. gingivalis was associated with tenderness to percussion in both deciduous and permanent teeth (p = 0.01, 0.015). The results of this study confirm that certain species of microorganisms are associated with clinical signs and symptoms of endodontic disease in both deciduous and permanent teeth.103

Gomes GB et al (2013) evaluated the frequency of selected endodontic pathogens in the pulp chambers (PCs) and root canals (RCs) of infected primary teeth using PCR methods. Paired PC and RC samples were collected from 15 subjects and analyzed by PCR for the presence of Filifactor alocis, Fusobacterium nucleatum, Parvimonas micra, Porphyromonas endodontalis, Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens, Prevotella tannerae, Tannerella forsythia, Treponema denticola, and Treponema socranskii. The species most frequently detected in PCs were P. nigrescens (86.7%), P. gingivalis (73.3%), and F. alocis (73.3%). Of the PC samples, 13.3% contained P. micra and T. denticola, and 6.7% contained T. forsythia. The species most frequently detected in RCs were P. gingivalis (100%) and P. nigrescens (93.3%). P. tannerae, P. micra, and T. denticola were found in 40% of the RC samples; T. forsythia was found in 26.7% of the RC samples. The "red complex", which comprises P. gingivalis, T. denticola, and T. forsythia, was not found in the PC of any tooth but was found in 30% of the RC
samples. The results suggest high heterogeneity among the samples, even among those from the same subject.\textsuperscript{104}

Fabris A et al (2014) evaluated the bacterial presence alone or in association in necrotic pulp and fistula samples from primary teeth of boys and girls. A predominance of gram-positive cocci (81.8\%) and gram-negative coccobacilli (49.1\%) was observed. In 88 out of 103 pulp samples, a high prevalence of \textit{Enterococcus} \textit{spp.} (50\%), \textit{Porphyromonas gingivalis} (49\%), \textit{Fusobacterium nucleatum} (25\%) and \textit{Prevotella nigrescens} (11.4\%) was observed.\textsuperscript{1}

3. Conclusion:

Recent developments in molecular methods have revolutionized the detection and characterization of microorganisms in dentistry. Among these methods, Polymerase Chain Reaction (PCR) has generated great benefits and allowed scientific advancements. PCR is an excellent technique for the rapid detection of pathogens, including those difficult to culture.\textsuperscript{108} Also, culture and PCR both suggest the polymicrobial nature of endodontic infection of deciduous teeth and predominance of anaerobes.
1.3 Justification for the study:-

A recent study has shown that high concentrations of antibiotics have a detrimental effect on human stem cells of the apical papilla (SCAP) survival. The exact reaction of the pulp to these drugs is not known. Hence, some histologic studies revealing the effects of these medicaments on the pulp tissue are needed.\textsuperscript{54} Other concerns are:

- The use of antibiotic can cause development of bacterial resistance.
- Already resistant microbes may be selected for and a superinfection may result;
- The antimicrobial may promote microbial chromosomal mutations;
- Gene transfer may be encouraged from resistant to nonresistant microbes; and
- Latent resistance genes may be expressed.\textsuperscript{66}

It is not advocated in a child especially with congenital heart disease or is at risk of infective endocarditis. Also, the potential sequelae of this treatment should be considered such as the risk of damage to the developing successor, cyst formation if a focus of chronic infection is left. In addition, localized minocycline staining of the permanent tooth bud may occur due to the excellent distribution of 3 Mix-MP.\textsuperscript{56}

Hence, the need to evaluate this technique which is extremely efficient but has concerns associated with it. As the prime concern is development of microbial resistance in relation to broad spectrum antibiotic, the use of these drugs should be avoided.\textsuperscript{104,105} So, different combinations of narrow spectrum drugs with different vehicles is attempted and it’s clinical efficacy will be checked.

Also, Chlorhexidine an effective broad-spectrum antimicrobial drug is being attempted at due to it’s unique properties of substantivity, safety and its inherent advantage over antibiotics by not producing resistant microorganisms.\textsuperscript{16,25}
Recently its modification i.e. Double Antibiotic Paste (DAP) consisting of Ciprofloxacin and Metronidazole with polyethylene glycol presented antimicrobial activity against 23 tested bacterial strains as intra-canal substances in an in vitro study. This study has shown that by the application of the experimental model, Ciprofloxacin and Metronidazole association with polyethylene glycol was the most effective combination for reducing the tested bacteria and yeasts.\textsuperscript{106}

This further modification of Double Antibiotic paste or Chlorhexidine might be equally successful and with fewer adverse effects and later, can be adopted as a intracanal medicament for deciduous teeth with necrotic pulp and high bacterial load. Thus, this innovative technique which has numerous applications can be adopted without jeopardizing the health of children. However, these antibiotics have their own advantageous contributions and side effects, with the eventual drug resistance as the most catastrophic consequence. Hence, an impending need is upon the fraternity of dentistry to find a reasonable alternative, which can best adapt to primary tooth physiology with maximum clinical benefit.

**Selection of Antimicrobials:**

1. Amoxicillin and clavulanic acid is one of the most effective antibiotic against oral microflora which consists of gram positive and gram negative obligate and facultative anaerobes including resistant *Enterococci* and β-lactamase-producing bacteria.\textsuperscript{107}

2. Metronidazole as infected root canals of teeth with abscess have predominant obligate anaerobes.\textsuperscript{13,108}

3. Chlorhexidine (CHX) has a broad spectrum activity against a wide array of oral microbes, high substantivity and capacity of inhibiting dentin matrix metalloproteinases.\textsuperscript{16}
4. Also, as per Principles of Antibiotic therapy; prudent combination of two bactericidal drugs and that too narrow spectrum drugs should be used to overcome the global menace of antibiotic resistance.¹⁰⁹

Selection of Vehicles:

1. The vehicle Polyethylene glycol showed antimicrobial effect and allowed greater penetration of the association with different intra-canal medicaments.¹⁰⁶

2. The vehicle Propylene glycol delivered dye through the root canal system rapidly and more effectively indicating its potential use in delivering intracanal medicaments.⁸¹

3. Glycerine as it is readily available at all pharmacies.

Selection of Micro-organisms:

A) FOR SUSCEPTIBILITY TO ANTIMICROBIAL DRUG COMBINATIONS:

1. *Streptococcus spp*; one of the pre-dominant species almost making upto 85% of the cultivable microflora of infected root canals by microbial culture.⁹⁸

2. *Porphyromonas gingivalis*; as most commonly isolated obligate anaerobe and many studies have shown an association between black-pigmented bacteria and endodontic infections.¹,⁹⁷

3. *Enterococcus faecalis*; is found in both primary and secondary endodontic infections in Deciduous teeth and is a virulent organism held responsible for failure of endodontic therapy.¹⁰²,¹¹⁰
B) FOR POLYMERASE CHAIN REACTION:

1. *Porphyromonas gingivalis*; has shown contrary findings in the two studies in deciduous teeth using polymerase chain reaction.\(^{103,111}\)

2. *Treponema denticola*; as they are difficult to grow and identify through microbial cultures.\(^{112}\)
AIMS AND OBJECTIVES

1.4 RESEARCH HYPOTHESIS

To test the equality of various combinations of intracanal medicaments and vehicles on selected pathogens from deciduous molars with necrotic pulp.

1.5 AIMS AND OBJECTIVES

Aim of the study: - To test the antimicrobial activity of various combinations of intracanal medicaments and vehicles on selected pathogens from deciduous molars with necrotic pulp.

Objectives of the study:-

PRIMARY OBJECTIVE

1. To evaluate and compare the antibacterial activity of;

   Group A: Ciprofloxacin and Metronidazole (Double Antibiotic Paste-DAP) with glycerine,
   Group B: Ciprofloxacin and Metronidazole (Double Antibiotic Paste-DAP) with polyethylene glycol,
   Group C: Ciprofloxacin and Metronidazole (Double Antibiotic Paste-DAP) with propylene glycol,
   Group D: Amoxicillin with clavulanate plus Metronidazole (Modified Double Antibiotic Paste-Modified DAP) with glycerine,
   Group E: Amoxicillin with clavulanate plus Metronidazole (Modified Double Antibiotic Paste-Modified DAP) with polyethylene glycol,
   Group F: Amoxicillin with clavulanate plus Metronidazole (Modified Double Antibiotic Paste-Modified DAP) with propylene glycol,
Aims & Objectives

Group G: Chlorhexidine gluconate with glycerine,

Group H: Chlorhexidine gluconate with polyethylene glycol and

Group I: Chlorhexidine gluconate with propylene glycol on the selected pathogens from infected root canals of deciduous molars.

SECONDARY OBJECTIVE

1. To identify selected microorganisms from root canals of infected deciduous molars by means of microbial culture. (*Streptococcus* spp, *Enterococcus faecalis* and *Porphyromonas gingivalis*)

2. To detect difficult to grow microorganisms from root canals of infected deciduous molars by means of Polymerase Chain Reaction. (*Enterococcus faecalis*, *Porphyromonas gingivalis* and *Treponema denticola*)
MATERIALS AND METHODS

The present research was conducted during the time period from May 2014 to July 2015 on 5-8 year old patients attending the Department of Pedodontics and Preventive Dentistry, KLE VK Institute of Dental Sciences, Belagavi. The present experimental study was conducted in 2 phases. The Phase 1 consisted of the procurement of materials required for the study followed by in vitro bactericidal activity of vehicles using broth dilution method against American Type Culture Strains (ATCC) strains. Later, in vitro antibacterial activity of innovative endodontic medicaments and different vehicle combinations using agar well diffusion against ATCC strains was done using pure and commercial drugs. The Phase 2 consisted of a pilot study before the recruitment of first subject followed by the main study which was conducted on children selected based on the inclusion and exclusion criteria.

The main study comprised of two parts pertaining to the primary and secondary objectives. The primary objective of the research was assessment of the antibacterial activity of the three intracanal medicaments, i.e. DAP, modified-DAP and Chlorhexidine with three vehicles, namely Glycerine, PEG and PG against three selected endodontic microorganisms using an Ex-vivo model. The selected endodontic pathogens (Streptococcus spp, Enterococcus faecalis, Porphyromonas gingivalis) were isolated from deciduous molars with necrotic pulp. The endodontic sample collection procedure, its culture, and identification followed by the Ex-vivo antibacterial activity assessment was standardized. The same endodontic sample was used for the secondary objective of research following standardization of DNA extraction procedure and PCR assay for detection of the specified organisms i.e. Enterococcus faecalis, Porphyromonas gingivalis and Treponema denticola.
Materials & Methods

Phase 1: *In vitro* studies against ATCC strains:

A. Determination of Bactericidal activity of vehicles by broth dilution
1. Propylene glycol (PG)
2. Glycerine
3. Polyethylene glycol (PEG) 400
4. PEG 1000
5. PG + PEG 400

B. Antibacterial activity of endodontic medicaments and vehicle combinations against selected microorganisms using pure drugs
1. *Streptococcus mutans*
2. *Staphylococcus aureus*
3. *E. faecalis*
4. *P. gingivalis*

C. *In vitro* antibacterial activity of innovative endodontic medicaments and different vehicle combinations using commercial
1. *Streptococcus mutans*
2. *Staphylococcus aureus*
3. *E. faecalis*
4. *E. coli*

Phase 2: Main study:

A. Primary Objective: *Ex-vivo* evaluation of Antimicrobial activity of various combinations of intracanal medicaments and vehicles

Intracanal Medicaments + Vehicles → Clinical Isolates of

- **DAP (C+M)**
- **Modified DAP (A+M)**
- **CHX**

- **Glycerine**
- **PEG 400**
- **PG**

Streptococcus spp
Porphyromonas gingivalis
Enterococcus faecalis

B. Secondary Objective (Polymerase Chain Reaction)

Detection of *E. faecalis* from endodontic samples by Conventional PCR
Detection of *P. gingivalis* and *Treponema denticola* by Multiplex PCR
Study design: Experimental study

Phase 1

In vitro Determination of Bactericidal activity of vehicles against ATCC strains

In vitro Antibacterial activity of endodontic medicaments and vehicle combinations against ATCC strains using pure and commercial drugs

Phase 2

Pilot study

Main study

Step I

Outpatients with the inclusion criteria, parental consent, and assent

Step II

Sample collection done and transported in RTF to BSRC

A. Primary Objective

Microbiological culture

Microbial Isolation and Identification

Antimicrobial activity using ex-vivo model against:
1) Streptococcus spp
2) Enterococcus faecalis
3) Porphyromonas gingivalis

B. Secondary Objective

Polymerase Chain Reaction

DNA Isolation

Gel Documentation using Agarose gel Electrophoresis:
1) Enterococcus faecalis
2) Porphyromonas gingivalis
3) Treponema denticola
SCHEMATIC REPRESENTATION OF THE METHODOLOGY

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<th>Phases of study</th>
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<td><strong>Phase 1</strong></td>
<td>➢ <em>In vitro</em> bactericidal activity of propylene glycol, glycerine, polyethylene glycol 400, and polyethylene glycol 1000 against selected microorganisms. (November - December 2013)</td>
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<td>➢ <em>In vitro</em> antibacterial activity of innovative endodontic medicaments and different vehicle combinations using pure and commercial drugs. (December 2013 - January 2014)</td>
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<tr>
<td><strong>Phase 2</strong></td>
<td>➢ Pilot study was conducted for standardization of sample collection, culture, antimicrobial activity assessment and DNA isolation. (January - March 2014)</td>
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<tr>
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<td>➢ <em>Ex-vivo</em> antimicrobial activity of various combinations of intracanal medicaments and vehicles on selected pathogens from deciduous molars with necrotic pulp. (May 2014 - June 2015)</td>
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<td>➢ Detection of <em>E. faecalis</em>, <em>P. gingivalis</em> and <em>Treponema denticola</em> from the same endodontic samples by PCR. (June 2015 - July 2015)</td>
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</table>

Sample Size Estimation:
Considering 95% confidence interval and 5% tolerable error and Power of the test at 80; sample size will be calculated using formula:

\[
n = \frac{Z^2 \times q}{0.01 \times p} = \frac{(1.96)^2 \times q}{0.01 \times p} = \frac{4 \times 20}{0.01 \times 80} = 96
\]

Where, \(Z\) stands for Standard Normal Variate.
METHOD OF COLLECTION OF DATA:

I. Procurement of Pure drugs, Vehicles and Microbiological materials:

The pure drugs, different vehicles and bacteriological culture media required for preliminary Minimum Inhibitory Concentration (MIC) studies were procured from HiMedia (Brucella agar base, Brain Heart Infusion, Enterococcus Confirmatory Hiveg Agar etc), and gift samples of Chlorhexidine gluconate 20% from Unilab Chemicals and Pharmaceuticals Ltd; by submitting the letter duly signed by guide. The purchase order was placed for the kits required for further biochemical parameters, DNA isolation and PCR assay. ATCC strains of Streptococcus mutans ATCC 25175, Staphylococcus aureus ATCC 12598, Enterococcus faecalis ATCC 35550, Porphyromonas gingivalis ATCC 33277, and Eschericia coli ATCC 25922 were procured for the in vitro studies of Phase 1. Treponima denticola ATCC 35405 was procured for the PCR assay i.e. Phase 2 of research protocol.

Materials:

The following instruments and supplies were used in sufficient numbers and quantity:

Phase 1:

A. In vitro Determination of Bactericidal activity of vehicles against ATCC strains by broth dilution

- Conical flasks (Borosil, India)
- Brain Heart Infusion (BHI) Broth (HiMedia, India)
- Distilled Water (Nice, India)
- Measuring cylinder
- Digital Weighing balance (Kern KB)
- Polyethylene glycol(PEG) 400 and 1000 molecular weight,
Materials & Methods

- Glycerine, and Propylene glycol (IP grade)
- Eppendorf tubes (Eppendorf)
- Micropipettes (0.5 to 10 microlitre, 10-100 microlitre) (Eppendorf)
- Micropipettes tips (Eppendorf)
- BHI agar (HiMedia, India)
- Glass Petri dishes (Steriplan-Duran, Reveira, Kruger and Brent)
- Sterile Inoculating loops
- Streptococcus mutans ATCC 25175, Staphylococcus aureus ATCC 12598, Enterococcus faecalis ATCC 35550 and Eschericia coli ATCC 25922
- Bacteriological Incubator (Bio-Technics India)

B. In vitro Antibacterial activity of endodontic medicaments and vehicle combinations against ATCC strains by agar well diffusion method using pure drugs

- Armamentarium for determination of MIC of antibiotics and their combinations by broth dilution method was same as that specified above.
- Amoxicillin with clavulanic acid (GlaxoSmithKline Pharmaceuticals)
- Ciprofloxacin and Metronidazole - pure form (Pharmacy college Store)
- 20% Chlorhexidine gluconate solution (Unilab, India)
- BHI agar (HiMedia, India)
- Glass Petri dishes (Steriplan-Duran, Reveira, Kruger and Brent)
- Sterile Inoculating loops (HiMedia, India)
- Micropipettes (0.5 to 10 microlitre, 10-100 microlitre)(Eppendorf)
Materials & Methods

- Micropipettes tips (Eppendorf)
- Measuring scale for Zones of Inhibition (HiMedia)
- All phases needed Vertical (Quesst International, USA) and Horizontal (Yorco, China) Laminar Airflow
- Four standard strains of organisms i.e. *Streptococcus mutans* ATCC (American Type Culture Collection) 25175, *Staphylococcus aureus* ATCC 12598, *Enterococcus faecalis* ATCC 3550, *Porphyromonas gingivalis* ATCC 33277 and *Eschericia coli* ATCC 25922

C. **In vitro Antibacterial activity of innovative endodontic medicaments and different vehicle combinations against ATCC strains by agar well diffusion method using commercial drugs**

- Armamentarium for determination of antibacterial activity of antibiotics and their combinations by agar well diffusion method was same as that specified above except for the commercial drugs.
  - Tablet Amoxicillin with clavulanic acid 325 mg (Mankind Pharmaceuticals)
  - Tablet Ciprofloxacin (Cipla Pvt Ltd)
  - Tablet Metronidazole (Abott Pvt Ltd)
  - 2% of Chlorhexidine gluconate solution (Dentachlor, Ammdent- India)
  - Scalpel no. 15 and B P handle
  - Mortar and pestle
PROCEDURE:

Phase 1:

A. **In vitro Determination of Bactericidal activity of vehicles against ATCC strains by broth dilution**

This study was carried out in Dr Prabhakar Kore Basic Science Research Centre, KLE University, Belagavi. The susceptibility of these organisms to PEG 400, PEG 1000, Propylene glycol, glycerine, and combination of Propylene glycol with PEG 400 was assessed using Broth dilution assay as Minimum Inhibitory Concentration (MIC) determination can be readily converted to determine the Minimum Bactericidal Concentration (MBC). Triplicates were performed for each of the microorganisms. As there is no study in literature comparing the effect of vehicles only, hence bactericidal activity of commonly used vehicles for intracanal medicaments was determined to find out the vehicle with maximum bactericidal activity.

- Culture media: Brain Heart Infusion (BHI) broth for MIC and BHI agar for MBC.
- Test organisms: Four microorganisms were selected for the study - *Streptococcus mutans* ATCC 25175, *Staphylococcus aureus* ATCC 12598, *Enterococcus faecalis* ATCC 35550 and *Eschericia coli* ATCC 25922.
- Inoculum preparation: The log phase or growth method was performed as follows. Atleast 3-5 well-isolated colonies of the same morphological type were selected from a culture plate. The top of each colony was touched with a loop, and the growth was transferred into an eppendorf tube containing 4-5 ml of BHI broth. The broth culture was incubated at 35 °C for 5-6 hours until
uniform turbidity of the 0.5 McFarland standard was attained. The turbidity of actively growing broth culture, to obtain a final turbidity optically comparable to that of the 0.5 McFarland standard is adjusted with BHI broth. It is carried out visually by comparing the inoculum tube and the McFarland standard against a white card with contrasting black lines.115

- Broth dilution method: A total of ten tubes were taken and nine dilutions of vehicles were done with BHI for MIC and MBC. In the initial tube only 200 µl of vehicle was added. 200 µl of BHI broth was added for dilutions into the next nine tubes additionally. In the 2nd tube containing 200 µl of BHI broth 200 µl of vehicle was added. This was considered as 10\(^{-1}\) dilution. From 10\(^{-1}\) diluted tube to make 10\(^{-2}\) dilution; 200 µl was transferred to second tube. The serial dilution up to 10\(^{-8}\) dilution was repeated for each vehicle. 5 µl was taken from the maintained stock cultures of required organisms, and added into 2 ml of BHI broth. 200 µl of the above culture suspension was added in each of the serially diluted tube. The growth control contained only the media and culture suspension in the last tube. The tubes were kept for incubation at 37 °C for 24 hrs in bacteriological incubator and observed for turbidity116 (Photograph 1).

- Minimum Bactericidal Concentrations (MBC): All tubes not showing visible growth were subcultured on BHI agar along with the growth control, after recording the MIC and incubated at 37 °C for 24 hrs. The amount of growth was noted, absence of growth indicated that the whole inoculum was killed and this highest dilution showing 99.99% inhibition was recorded as MBC\(^{116}\) (Photograph 2).

Triplicates were performed for each of the microorganisms.
B. *In vitro* Antibacterial activity of endodontic medicaments and vehicle combinations against ATCC strains by agar well diffusion method using pure drugs

The endodontic medicaments evaluated were DAP i.e. Ciprofloxacin with Metronidazole, modified DAP i.e. Amoxicillin clavulanate with Metronidazole and 2% Chlorhexidine gluconate solution and their combination with Polyethylene glycol (PEG 400), Propylene glycol (PG), glycerine and combinations of PG with PEG. The antimicrobial activity was carried out against five commonly isolated microorganisms from root canals. American Type Culture Collection (ATCC) strains were utilized for this *in vitro* study. *Streptococcus mutans* is one of the most commonly isolated organism from root canals of infected teeth and considered to play a crucial role in pathogenesis of dental caries. Whereas *Staphylococcus aureus* and *Enterococcus faecalis* are known to be resistant species, and not being susceptible to the most common drug regimens. *Porphyromonas gingivalis* is predominant in necrotic root canals of teeth and related to the signs and symptoms of periapical disease. Gram negative obligate anaerobe are considered to be more resistant due to the outer membrane of their cell-wall structure and as in the case of *Porphyromonas gingivalis*. Agar well diffusion assay using pure drugs was done for the standardization of the procedure and evaluation of MIC of individual drugs and their combinations with vehicles.

**Preparation of Microbial Inocula:**

A direct colony suspension of each organism to be tested was prepared. The turbidity was adjusted to 0.5 McFarland standard, for *S.mutans*, *S. aureus*, *E. faecalis*, *E. coli* and a 1.0 Mcfarland standard for *P. gingivalis* as per Clinical
Laboratory Standard Institute (CLSI) guidelines formerly known as National Committee for Clinical Laboratory Standards i.e. NCCLS.\textsuperscript{116}

Determination of Minimum Inhibitory Concentration (MIC) of antimicrobial substances and their combinations by broth dilution method:

MIC is defined as the lowest concentration where no visible turbidity is observed. Hence, MIC can be referred to as the bacteriostatic concentration. Brain Heart Infusion (BHI) broth was used for the serial dilutions. The selected micro-organism was inoculated in BHI broth as per CLSI guidelines. In accordance to the CLSI guidelines for antimicrobial susceptibility testing; control strains of \textit{E. coli} ATCC 25922 was kept for monitoring. A known concentrate of the antibacterial substances was serially diluted to two folds in broth. Two controls were also maintained. The first control consisted of undiluted drug as the positive control and the negative control contained only inoculums. MIC was performed using broth dilution method first for single drug namely 2\% Chlorhexidine gluconate, Ciprofloxacin, Amoxycillin clavulanate, Metronidazole (Photograph 3) and their combinations i.e., Ciprofloxacin with Metronidazole(C+M) and Amoxycillin clavulanate with Metronidazole (A+M) (Photograph 4). The concentrations used were 0.5mg/ml of Ciprofloxacin, 0.5mg/ml of Amoxicillin clavulanate and 2mg/ml of Metronidazole for single drugs and 1mg/ml concentration for both the drug combinations i.e., C+M and A+M.\textsuperscript{116}

Determinations of antimicrobial susceptibility using agar well diffusion method:

The antibacterial activity of endodontic medicaments and various vehicles was determined using agar well diffusion method in terms of zones of inhibition. The endodontic medicaments tested were DAP, modified DAP and 2\% CHX with PEG, PG, glycerine and PEG+PG. BHI agar plates have been used for facultative
organisms' and Blood agar was used for obligate anaerobes i.e., *P. gingivalis* for agar well-diffusion method. A 50 µl of respective microbial inoculum was taken using a micropipette, loaded onto the agar plates and swabbed evenly to provide an uniform lawn of cells. The BHI and Blood agar plates were inoculated with the respective microorganisms by even streaking of the swab over the entire surface of the plate; three times rotating the petri plates at 60 degrees approximately after each application. Lastly, it was swabbed all around the periphery of the agar surface. Five wells of 7mm size and 4mm depth were made at an equal distance and 70µl volume (35 µl medicaments+35 µl vehicle) of each medicaments with respective vehicles in the ratio 1:1 was dispensed into the 4 wells with the help of micropipettes. The undiluted medicaments i.e., 70 µl of the antimicrobial substances only, was dispensed into the 5th well at the centre of inoculated agar plate and was considered as the positive control. The plates were then incubated at 37 °C for 24 hrs for *S. mutans*, *S. aureus* and *E. faecalis* in an aerobic environment and for *P. gingivalis* for 48 hrs anaerobically.

The petri plates were observed for zones of inhibition after overnight incubation in a bacteriological incubator. Zones were measured using a scale in millimeters (Photograph 5). The tests were repeated in triplicates.

C. *In vitro* Antibacterial activity of innovative endodontic medicaments and different vehicle combinations against ATCC strains by agar well diffusion method using commercial drugs

The selected microorganism i.e., *Streptococcus mutans* (ATCC 25175), *Staphylococcus aureus* (ATCC 12598), *Enterococcus facealis* (ATCC 35550) and control as *Escherichia coli* (ATCC 25922) were inoculated in BHI broth as per CLSI guidelines for antimicrobial susceptibility testing. Also control strain of *E. coli*
(ATCC 25922) was kept for monitoring antibacterial susceptibility testing. The agar well diffusion method was carried out using BHI agar plates.\textsuperscript{116} Agar well diffusion assay using commercially available drugs was carried out to standardize the amount of drugs required to be placed as intracanal medicaments to simulate the clinical situation. It also aided in comparison of antimicrobial activity assessment by means of agar well diffusion method and the \textit{ex-vivo} model.

**Preparations of Microbial Inocula:**

The turbidity of the direct colony suspension of each organism to be tested was adjusted to 0.5 McFarland standard, for \textit{S. mutans}, \textit{S. aureus}, \textit{E. faecalis}, \textit{E. coli} as per CLSI guidelines.\textsuperscript{116}

**Preparation of the various combinations of endodontic medicaments and vehicles:**

Commercially available antibiotics such as Ciprofloxacin, Metronidazole, and Amoxicillin Clavulanate and 2\% Chlorhexidine gluconate solution were used in the study. The removal of enteric coating of the tablets was done with a surgical scalpel \#15 blade with Bard Parker handle No.3 was used. The drugs were powdered using sterile porcelain mortar and pestle. These powdered drugs were stored in air-tight containers and refrigerated till use. Each drug in concentration of 0.5 mg each of Ciprofloxacin 400mg, Metronidazole 200mg and Amoxicillin clavulanate 325mg was taken using a weighing scale. To create a paste with proper thickness the DAP and modified DAP were mixed with respective four vehicles prior to placement in the wells made in BHI agar plates for agar well diffusion assay in 1:1:1 ratio.\textsuperscript{56,59,58}

1. **Preparation of DAP:** Ciprofloxacin, and Metronidazole will be taken in a ratio of 1:1.
2. **Preparation of Modified DAP:** Amoxicillin-clavulanate, and Metronidazole will be taken in a ratio of 1:1.

3. **Commercially available 2% Chlorhexidine gluconate (DENTACHLOR):**

   50 µl of 2% Chlorhexidine gluconate solution was be taken with a micropipette.

   These endodontic medicaments in the ratio of 1:1 were mixed with respect to one drop i.e., 10 µl of vehicles i.e., PEG 400, PG, glycerine and combinations of PG with PEG whereas 2% Chlorhexidine gluconate was mixed with 50µl of each vehicle.

**Determinations of antimicrobial susceptibility using agar well diffusion method:**

Agar well diffusion method was used to determine the antibacterial activity of endodontic medicaments and various vehicles i.e. DAP, modified DAP and 2% CHX with PEG, PG, PEG+PG and glycerine. The BHI agar plates were prepared similarly as mentioned earlier. Five wells of 6mm size and 4mm depth were made at an equal distance. A total of 10µl volume of each medicament pastes and 100µl of 2% CHX with respective vehicles in the ratio 1:1 was dispensed into the 4 wells with the help of micropipettes. 100 µl of sterile normal saline only, was dispensed into the 5th well at the centre of inoculated agar plate and was considered as the negative control. The plates were then incubated at 37 ° C for 48 hrs in an aerobic environment for *S. mutans, S. aureus* and *E. faecalis* while *E. coli* as a quality control strain.¹¹⁶

The petri plates were observed for zone of inhibition after overnight incubation, zones were measured using a scale in millimeters (Photograph 6, 7, and 8). The tests were repeated three times.
Phase 2: Pilot and Main Study

Materials:

A. Primary Objective-Antimicrobial activity of various combinations of intracanal medicaments and vehicles

- Mouth mirror
- Probe
- Tweezer
- Spoon excavator
- Sterile Suction tips (First Class, Italy)
- 2 ml syringe and needle (Unolok, Hindustan Syringes and Medical Devices Ltd., Faridabad)
- Topical Local anaesthetic (LidFAST, Deep Healthcare Pvt. Ltd., Ahmedabad)
- Local anaesthetic solution (2% lignocaine with 1:80,000 adrenaline. Xicaine, ICPA Health Products Ltd., Ankleshwar)
- Rubber dam kit (Sheet, Punch, Template, Clamp, Clamp holder, Frame) (Hygeinic Fiesta, Coltene Whaledent Inc., USA)
- Airotor handpiece (NSK PANA AIR 2 JAPAN, No. C3871893)
- Sterile round bur (MANI Inc., Japan)
- Hand #15 K files (MANI Inc., Japan)
- Sterile absorbent points (DiaDent Paper Points, China)
- Measuring scale
- Gloves (Rakshak, RamyaImpexPvt. Ltd., Mumbai)
- Head cap (Ramson’s Care Plus, Ramson Health Care, Bangalore)
- Face Mask (Ramson’s Care Plus, Ramson Health Care, Bangalore)
Materials & Methods

- Size ‘0’ X ray Film (Kodak Insight, Carestream Health Inc., NY, USA)
- Dental Floss (StimTuf- Floss, Global DentAidsPvt. Ltd., New Delhi)
- Cotton (Prabhat Surgical Cotton Pvt. Ltd., Tumkur, Karnataka, India)
- Cotton holder
- Reduced Transport Fluid (RTF) media
- Glass Petri dishes (Steriplan-Duran, Reveira, Kruger and Brent)
- Sterile Inoculating loops (HiMedia, India)
- Electric Loop Sterilizer (HiMedia, India)
- M-1039 Brucella agar base with hemin and Vitamin K – 500 gm (HiMedia, India)
- Horse serum RM-1239 – 100 ml (HiMedia, India)
- M-979, Thioglycollate broth with hemin and Vitamin K – 500 gm (HiMedia, India)
- Kanamycin sulfate 7c-36 – 1gm (HiMedia, India)
- Enterococcus Confirmatory Agar HiVeg™ MV 392-500gm (HiMedia, India)
- Distilled water (NICE, India)
- Measuring cylinder
- Anaerobic Jar (HiMedia, India)
- Carbon di oxide-CO₂/Candle Jar (Riveira, India)
- Sodium borohydride RM 10345 – 100gm (HiMedia, India)
- Citric Acid (anhydrous) ( Fisher scientific, Qualigens);and
- Sodium carbonate anhydrous GR(Merck)
- Palladium catalyst pellets (HiMedia, India)
- Vacuum Suction (Rivotek™, India)
• Bacteriological Incubator (Bio-Technics India)
• Gram Stains-Kit (HiMedia, K 001-1KT)
• Glass slides and cover slips (HiMedia, India)
• Stereomicroscope (Labomed Lx400, USA)
• Digital Weighing balance (Kern KB)
• Tablet Amoxicillin with clavulanic acid (Mankind Pharmaceuticals)
• Tablet Ciprofloxacin (Cipla Pvt Ltd)
• Tablet Metronidazole (Abott Pvt Ltd)
• Polyethylene glycol 400, Glycerine and Propylene glycol (IP grade)
• Chlorhexidine gluconate (Unilab India)
• Sterile swabs (PW005, Jay Distributors)
• Extracted deciduous molars
• Cavit™ (3M ESPE, UK)

B. Secondary Objective- PCR related Laboratory Armamentarium

• Enterococcus faecalis (ATCC® 35550)
• Porphyromonas gingivalis (ATCC®33277)
• Treponema denticola (ATCC®35405)
• Proteinase K (SE PK-10 mg/ml)
• PCR Master Mix (Ampliqon)
• Oligonucleotides primers (Bioserve India Pvt Ltd, Hyderabad)
• Eppendorf tubes (Eppendorf)
• Micropipettes (0.5 to 10 microlitre, 10-100 microlitre) (Eppendorf)
• Micropipettes tips (Eppendorf)
• Micro-centrifuge (Bio-bee)


**Materials & Methods**

- Waterbath (Bio-bee)
- Deep Freezer(-20 °C) (Blue Star)
- Vertical Laminar Airflow (Quesst International, USA)
- Veriti 96-Well Fast Thermal Cycler (Applied Biosystems, USA)
- Gel electrophoresis system (Bio-bee)
- Gel Documentation system G Box (Syngene, UK)
- Ethidium Bromide (Himedia, India)
- Agarose for gel preparation (Himedia, India)
- Computer (Lenovo)

**II. Approvals:**

The **Ethical Clearance Certificate** was obtained for the proposed activities as per the experimental protocol approved by **Institutional** and **PhD Human Ethical Committee**. The PhD Human Ethical Committee meeting held on 29th April 2014, and the research protocol, Informed consent, Assent form and the Proforma were approved.

Before collection of the samples from the first subject, the pilot study was approved by the Institutional Research and Ethical Committee followed by the PhD Human Ethical Committee Ref. No. KLEU/Ethic/14-15/D-73 approval. The Ethical Clearance Certificate from both the Institutional and PhD Ethical Committee are attached respectively (**Annexure-I and II**).

Informed written consent was obtained from all the parents of children participating in the study and approved Informed Consent Form is attached as **Annexure-III**.

Assent was obtained from all the children participating in the study and approved. Assent Form attached as **Annexure-IV**.
The approved Proforma is attached as Annexure-V.

Source of Data:

The study was conducted on patients seeking treatment at Department of Pedodontics and Preventive Dentistry, KLE VK Institute of Dental Sciences, Belagavi.

Selection of Subjects:

One hundred eleven endodontic samples were taken from 37 teeth (28 mandibular molars and 9 maxillary molars: three root canals per tooth) from 35 (15 female and 20 male) 5-8 year old children residing in Belagavi city and attending the outpatient department of Pedodontics and Preventive Dentistry, Belagavi, India. Informed consent was taken from each parent and assent was taken from children too. A proforma was recorded for each patient comprising of casehistory recording, along with investigations and diagnosis. Patients willing to participate in the study and fulfilling the inclusion criteria were included in the study.

INCLUSION CRITERIA:

1. Patient aged 5-8 years.
2. Deciduous molar teeth with necrotic pulp, chronic abscess and/or sinus tract.
3. Deciduous molar teeth with no gingival recession and free of periodontal pockets more than 2 mm deep.
4. Deciduous molars with almost 2/3rd of the roots present and no previous pulp therapy.
5. Compliant patients.
EXCLUSION CRITERIA:

1. Teeth with more than two third of tooth structure lost or tooth which could not be isolated with rubber dam.
2. Patients with antibiotic usage or antimicrobial mouthwashes for systemic diseases in past 3 weeks.
3. Teeth previously treated by any form of pulp therapy.
4. Teeth with failed pulp therapy and need re-treatment due to developed signs or symptoms.
5. Patient with systemic diseases and any history of drug allergy.
6. Unco-operative patients and patients or their parents not willing to accept proposed treatment plan and/or participate in the study.

PROCEDURE:

The Phase 2 comprised of Pilot study followed by the Main study related to the primary and secondary objectives:

➢ Pilot study:

   Pilot study was carried out by collecting five endodontic samples from deciduous molars with necrotic pulp after obtaining informed consent from parents of children included in the study. Assent was also taken from all children who participated in the study. A detailed medical and dental history was obtained from parents of children and the Proforma was filled accordingly. The purpose of the pilot study was to standardize the method of collection of biomaterial sample using #15 K files and sterile paper points, access opening and disinfection protocol prior to access opening. Standardization of the culture, sub-culture, isolation of selected
Materials & Methods

Microorganisms, antimicrobial activity testing and DNA isolation for PCR assay was also done. The pilot study samples were not included in the main study.

C. Standardization of Disinfection Protocol, Endodontic Biomaterial Sample Collection and Culture and Isolation:

- **Disinfection protocol** was adopted from Gondim et al (2012). Initial antisepsis of the oral cavity was carried out with 0.12% Chlorhexidine mouthwash or swabbed with in 0.12% Chlorhexidine swabs. After local anaesthesia, rubber dam was placed, and disinfection of the tooth surface and operative field with 1% Chlorhexidine. The disinfection protocol was chosen as it was less time consuming as compared to other protocols and practical one considering it was to be carried out in 5-8 year old children. To confirm effectiveness of disinfection samples were taken from tooth surfaces in every fifth sample and cultured on Blood agar. An indication of no growth was considered as successful disinfection.

- **Endodontic biomaterial sample collection** was adapted from Cogulu et al (2007). To standardize, access cavity preparation was done using sterile burs without water spray. Sterile saline drops were used as coolant during cavity preparation. Aseptic techniques were used for instrumentation and sample collection also, by using new file each time and for each canal. Each root canal was sampled separately in each tooth contributing to three samples per tooth to confine the microbiological evaluation to a single ecologic environment. Also, all the canals of a single tooth though diagnosed as necrotic with radiographic radiolucency in furcation area had few canals vital while; others non-vital. Hence, there ecologic environment might vary. Literature reveals that microbiota of vital and non-vital or necrotic canals is different with predominance of anaerobes in non-vital necrotic teeth. Also, root canals in
deciduous molars are anatomically well differentiated and isolated clinical structures, thus enhancing the chances of culturing from different environment of the three different root canals of the same tooth. Samples were collected with sterile # 15 K-file followed by two paper points. The # 15 K-file was used in a filing motion i.e., up and down motion to agitate the contents of the canal for 30 secs after insertion to the depth of the working length followed by two paper points sequentially inserted for 30 secs each to soak up the contents within the root canal. The handle of #15 K file was cut off after sample collection\textsuperscript{122,123,124} and transferred to the Eppendorf tube containing Reduced Transport Fluid(RTF). The two paper points inserted in the same canal were also transferred to the same Eppendorf tube to be sent to laboratory for further processing.\textsuperscript{122,123,124} If the canal was dry, a small amount of RTF was added drop by drop into the dry canal to avoid flooding but at the same time, make sample collection feasible.\textsuperscript{111} Thus, endodontic biomaterial sample was taken and transferred to eppendorf tube containing 2 ml RTF. This sample collection protocol was arrived upon when initial sample collection with only paper points failed to give results during the Biophotometric readings taken after DNA isolation, hence # 15 K files and RTF were appropriately incorporated into the sample collection protocol. RTF was used as transport media as many studies have proved beyond doubt that it is a efficient medium for the transport of oral bacteria especially anaerobes in the samples.\textsuperscript{125,126,127} After this protocol was adopted, the Pilot study samples gave readings of quantification of DNA i.e., the A260/A280 ratio in the range of 1.6-1.8 indicating good purity of DNA.
• **Standardization of Culture and Isolation:**

Clinically isolated strains of *Streptococcus spp*, *Porphyromonas gingivalis*, and *Enterococcus faecalis* were utilized instead of ATCC strains to get the existing antibiotic susceptibility of clinical strains of microorganisms and to formulate clinical guidelines regarding the ideal antibiotic drug to be prescribed when indicated for oral infections.

1. *Streptococcus spp*:

   To standardize the culture and isolation process, endodontic samples collected during the pilot study were streaked on blood agar and incubated for 37°C for 48 hrs in anaerobic and candle jar. The CFUs were counted and pure colonies were picked and gram staining was done in order to confirm *Streptococcus spp*. Once *Streptococcus spp* was confirmed pure colonies were picked and subcultured to get pure growth against which antimicrobial susceptibility would be carried out using *ex-vivo* model.

2. *Porphyromonas gingivalis*:

   To standardize the culture and isolation process, endodontic samples collected during the pilot study were streaked on Kanamycin blood agar and incubated for 37°C for 72 hrs in anaerobic and candle jar. The CFUs were counted and pure colonies were picked and gram staining was done in order to confirm *Porphyromonas gingivalis*. These were further confirmed using sugar tests. Once *Porphyromonas gingivalis* was confirmed pure colonies were picked and subcultured to get a pure growth; against which antimicrobial susceptibility was going to be carried out using *ex-vivo* model.
3. *Enterococcus faecalis*:

This study is the first study utilizing Enterococcus Confirmatory HiVeg Agar (ECA). Enterococcus Confirmatory Agar was selected for detection of *Enterococcus faecalis* as it is a more specific media to avoid false positive detection of colonies as *E. faecalis* also grows and can be identified on blood agar. Colony characteristics of *Enterococcus faecalis* ATCC 29212 shown on Enterococccus Confirmatory HiVeg Agar were assessed:

- *Enterococcus faecalis* ATCC 29212 was streaked on Enterococccus Confirmatory HiVeg Agar to note colony characteristics.
- After streaking the plates were incubated at 37 °C for 48-72 hours in CO₂ dessicator.
- Colony characteristics were noted.
- *Klebsiella pneumoniae* growth observed on streaking ECA where as *Escherchia coli* no growth observed.
- To standardize the culture and isolation process, endodontic samples collected during the pilot study were streaked on ECA agar and incubated for 37 °C for 72 hrs in candle jar. The CFUs were counted and pure colonies were picked and gram staining was done in order to confirm *Enterococcus faecalis*. Once *Enterococcus faecalis* was confirmed pure colonies were picked and cultured in BHI broth to get a pure growth against which antimicrobial susceptibility was going to be carried out using *ex-vivo* model.

- **Standardization of Antimicrobial activity assessment using *ex-vivo* model:**

An innovative *ex- vivo* model was developed and utilized inorder to simulate as closely as possible the clinical situation of necrotic deciduous teeth in children’s
oral cavity. Intracanal medicaments like Calcium hydroxide are ineffective due to presence of buffers, tissue debris and inability to penetrate the dentinal tubules. Hence, testing the efficacy of intracanal medicaments in situ; is crucial to predict its efficacy clinically. A sufficient number of extracted, multi-rooted human deciduous teeth, including primary maxillary and mandibular molars with almost 2/3rd of the roots present were selected for the study. The teeth were cleaned with periodontal curettes to remove periodontal tissues and bone and stored in saline. The teeth were then sterilized in autoclave at 121°C and 15 psi for 20 minutes. Access cavity preparation was done using sterile round burs with air-rotor under continuous water cooling. After access cavity preparation the extracted deciduous molars were again stored in saline and were subjected to sterilization by autoclave prior to their use in the ex-vivo model.

Initially antimicrobial activity assessment was done against ATCC strains of Streptococcus mutans, Enterococcus faecalis and Porphyromonas gingivalis. Initial antimicrobial tests were carried out in Micropipette Tip boxes and later petri plates of glass from Steriplan-Duran, Reveira, Kruger and Brent were found to be ideal; allowing the teeth and it's root length to be immersed in the agar.

Since placement of 2% Chlorhexidine solution was inappropriate as an endodontic medicament, decision of using 2% Chlorhexidine gel as it is the commonly used intracanal medicament was made. Due to inavailability of commercial 2% Chlorhexidine gel in India, it was prepared using hydroxypoly methyl cellulose (HPMC ) polymer in the Department of Pharmaceutics, College of Pharmacy, KLE University, Belagavi.
• **Standardization of DNA isolation and gel documentation:**

The investigator was trained and calibrated for DNA isolation procedure under the research supervisor following attending a workshop in the Dr. Prabhashar Kore Basic Science Research Centre (*Annexure-VI*). The sample collection protocol was improvised to increase the purity of isolated DNA, until the Biophotometric quantification readings of the A260/A280 ratio came in the range of 1.6-1.8. The isolated DNA was later amplified and gel documentation was carried out to confirm the organisms we had isolated. Lysosome was added to the protocol to improve gram negative cell wall disruption and DNA isolation consequently.

• **Standardization of Proforma:**

A proforma was prepared to probe regarding the demographic details, medical, dental and drug history, and personal history. Proforma alongwith the case history also included oral soft tissue and hard tissue findings, investigations if recommended and Final Diagnosis to formulate the complete treatment plan (*Annexure-V*). Details of five subjects were collected and the recording of the proforma was assessed and Informed consent and Assent were introduced.

• **Standard Operating Procedures:**

SOP’s were developed for Digital weighing balance, Laminar airflow unit, Anaerobic jar, Labotech Bacteriological Incubator, Hot air Oven and Agar composition and preparation procedures and lastly, Gram staining (*Annexure-VII*).
Main Study:

A. Primary Objective (Microbial culture and Antimicrobial activity using \textit{ex-vivo} model):

The primary objective of research consisted of assessment of the antibacterial activity of the three intracanal medicaments, i.e. DAP (C+M), modified-DAP (A+M) and Chlorhexidine (CHX) with the three vehicles, namely Glycerine, PEG and PG against three selected endodontic microorganisms using an \textit{ex-vivo} model. These three vehicles were the most effective and the combination of PEG and PG being least effective; as was evident in the previous \textit{in vitro} studies. Hence, these three vehicles were selected for the main study. The selected endodontic pathogens (\textit{Streptococcus spp, Enterococcus faecalis, Porphyromonas gingivalis}) were isolated from 5-8 year old patients attending the Department of Pedodontics and Preventive Dentistry, KLE Vishwanath Katti Institute of Dental Sciences, Belagavi; diagnosed having deciduous molars with necrotic pulp. All samples were collected by an experienced pediatric dentist who was also the principal investigator of this experimental research protocol.

B. Secondary Objective (Polymerase Chain Reaction):

The secondary objective of research consisted of PCR detection of the specified organisms i.e., \textit{Enterococcus faecalis, Porphyromonas gingivalis} and \textit{Treponema denticola} from the same endodontic sample collected from deciduous molars with necrotic pulp following DNA extraction procedure.
A. Primary Objective - Antimicrobial activity of various combinations of intracanal medicaments and vehicles:

Step I

Outpatients with the inclusion criteria reporting to the Department of Pedodontics and Preventive Dentistry at the KLE VK Institute of Dental Sciences were selected for the study (Photograph 9), informed consent from parents and assent from children taken. Proforma was filled; and preoperative intraoral periapical radiographs was taken.

Step II

Initial antisepsis of the oral cavity was carried out with 0.12% CHX mouthwash or swabbed with 0.12% Chlorhexidine swabs.24,95

- Local anaesthesia was administered using 2% lignocaine with 1:80,000 adrenaline.
- Rubber dam isolation was done of the single tooth which was diagnosed to be necrotic after taking a pre-operative diagnostic radiograph (Photograph 10).
- Antisepsis with 1% Chlorhexidine digluconate was done.24,95
- Standard access cavity preparation was done using a sterile round bur.
- The patency of the canals were checked with a #15 K file upto the working length assessed from the pre-operative radiograph (Photograph 11).
- The # 15 K-file was used in a filing motion i.e., up and down motion to agitate the contents of the canal for 30 secs after insertion to the depth of the working length followed by two paper points sequentially inserted for 30 secs each to soak up the contents within the root canal.
The handle of #15 K file was cut off after sample collection and transferred to the Eppendorf tube containing reduced transport fluid i.e. RTF (transport medium). 

Absorbent points were taken into the canal till the apex and were held there till 30 seconds, each one following another to absorb all the fluid in the root canal (Photograph 12). If the canal was dry, a small amount of RTF was added drop by drop into the dry canal to avoid flooding but at the same time, make sample collection feasible.

The absorbent points were then removed and transferred directly into a tube containing RTF (Photograph 13).

This tube was taken to the Dr. Prabhakar Kore Basic Science Research Centre where it was processed within 2 hours of sample collection for culture and PCR assay.

Reduced transport fluid in eppendorf tubes supplemented was used as transport medium. It was collected from BSRC as soon as a patient was recruited for the study and consent was taken; prior to sample collection.

After thoroughly shaking the endodontic biomaterial sample in a mixer for 60 secs, a part (required amount) of each sample were used for culture and the rest of the sample was frozen immediately at -20 °C until assayed by PCR.
Step III

For Microbial Isolation and Identification:

- Samples were processed microaerobically by the candle (CO₂) jar system (Photograph 14) and anaerobically inside an anaerobic chamber (85% N₂, 10% H₂, 5% CO₂) by evacuation replacement procedure (Photograph 15).

- In the evacuation replacement, anaerobiosis was achieved by the formation of the hydrogen and carbon dioxide gas mixture using a mixture of 500mg of sodium carbonate, 500mg of citric acid and 500mg of sodium borohydride.

- The liberated hydrogen combines with oxygen in the presence of palladium catalyst to form water. At the same time, traces of carbon di oxide released from the first reaction stimulate growth of anaerobic bacteria.

- Each sample was inoculated on a 5% sheep-blood agar plate with Vitamin K and Haemin in a CO₂ jar observed after 48 hours, a 5% sheep-blood agar plate with Vitamin K and Haemin along with a 5% sheep-blood agar plate with Kanamycin incubated anaerobically and Enterococcus Confirmatory Agar incubated at 37 °C in CO₂ jar, for 72 hours each till growth was observed.¹³¹

- Colonies were counted in CFUs and pure colonies were picked and gram staining was done in order to confirm the targeted microorganism.

- Strains were identified based on Gram staining and classified by colony morphology, oxygen tolerance and biochemical test.¹³¹

- These isolated pure colonies were picked up using a sterile nichrome loop and subcultured to get pure colonies for ex-vivo tests.

- All isolated microbial strains of Streptococcus spp, Enterococcus faecalis, Porphyromonas gingivalis were subjected to antimicrobial susceptibility testing using ex-vivo model.
Materials & Methods

- Treatment was completed in the children from whose teeth biomaterial has been collected in the Department of Pedodontics and Preventive Dentistry.

Microbial Analysis:

- The endodontic biomaterial sample was vortexed for uniform mixing of the sample with RTF.
- The media and incubation done for the culture were:
  a. Blood agar for *Streptococcus spp* incubated in CO$_2$ jar for 48 hours (Photograph 16).
  b. Blood agar (Photograph 17) and Kanamycin blood agar (Photograph 19) for *Porphyromonas gingivalis* incubated in anaerobic jar for 72 hours.
  c. Enterococcus Confirmatory Agar for *Enterococcus faecalis* incubated in CO$_2$ jar for 72 hours (Photograph 18).

**Procedure for Streak culture and incubation**

- The sample in the centrifuge tube was vortexed for 30 seconds.
- Vertical laminar flow was prepared by wiping with 70% alcohol and switching on the ultraviolet light for 15 minutes.
- Sterile agar plates were taken out of the refrigerator and brought to normal temperature.
- After this they were labelled around the perimeter at the bottom of the agar plate for the name, sample no., date and divided into three parts to accommodate all the three samples from the three root canals of the same tooth. This was to have a direct comparison of the microbes isolated from the three different canals of the same tooth.
Materials & Methods

- Nichrome loop of 2-4 mm diameter was used for streaking the sample and quadrant streak method was used.

- Loop was first sterilized in electric loop sterilizer by making it red hot and cooled by touching an another spare sterile agar plate.

- 5 – 10 µl of sample was taken with the help of micropipettes and added near the peripheral area of the culture plate.

- With the help of loop, it was smeared onto the surface of dried plate near peripheral area. This is termed as primary inoculums and its streaking.

- From the primary inoculum, that is intersecting the primary streaks at approximately 90º, it was streaked in parallel lines over the plate with the help of sterile loop.

- Lastly, tertiary streaks were given from secondary ones followed by formation of a tail. This is done for uniform distribution of the organisms in the sample.

- The loop was flamed and cooled in between different sets of streaks.\textsuperscript{115}

- Same procedure was done for all the 3 media.

- Blood agar petri plates were placed in inverted position for incubation in CO\textsubscript{2}/candle jar to avoid condensation on the lid and drip onto the culture, causing colonies to spread into each other for 48 hrs.; and

- Blood agar and Kanamycin blood agar petri plates were placed inverted and incubated in anaerobic jar (Modified McIntosh-Fildes jar) for 72 hours.

- ECA agar plates were incubated in CO\textsubscript{2} jar for 72 hours.

- After 48-72 hours, both the jars were opened and checked for the growth of the respective microorganisms.\textsuperscript{131}

- Colonies were counted and results expressed as Colony forming units/ml.
Confirmation of microorganisms

Confirmation of *Streptococcus spp, P. gingivalis and Enterococcus Faecalis* by gram staining:

- Gram staining procedure and the SOP developed are attached in Annexure-VII. Gram staining was done for confirmation of *Streptococcus spp*, *P. gingivalis* and *Enterococcus faecalis* initially followed by biochemical tests.
- Two or three colonies from the culture were picked and spread over the glass slide containing normal saline.
- Saline and pure colonies were mixed and smear was prepared with the nichrome loop.
- Glass slide was heat fixed by flaming over spirit lamp.
- Smear was flooded with crystal violet.
- After 60 seconds, glass slide was washed with running water.
- Smear was flooded with Gram’s iodine for 60 seconds.
- Slide was washed with running water.
- 70% alcohol was added and kept for 30 seconds.
- Slide was washed with running water.
- Counterstain (Safranine) was added and kept for 30 seconds.
- Slide was washed with running water and dried.
- Slide was observed under stereomicroscope (100x) in oil immersion.
- Respective microorganism (*Streptococcus spp, P. gingivalis* and *Enterococcus faecalis*) were identified (Photograph 20).
- *Streptococcus spp* were identified as gram positive cocci in chains.
Materials & Methods

- *Porphyromonas gingivalis* were identified as gram negative, rod-shaped or cocci-bacilli.

- *Enterococcus faecalis* were identified as gram positive diplococci or short chains.

- *Enterococcus faecalis* was specifically cultured on Enterococcus Confirmatory Agar, which was further confirmed by gram staining too.

*P. gingivalis*

- Two or three colonies were taken from the petri plates and added to eppendorf tube containing thioglycollate broth and horse serum and incubated for 72 hours in the anaerobic jar.

- The eppendorf tube was removed from the anaerobic jar after 72 hours and sugar fermentation tests were performed.

- Sugar fermentation tests were done to differentiate between *Porphyromonas gingivalis* and *Prevotella intermedia*.

- Ten µl of inoculum from the centrifuge tube was added to the centrifuge tubes containing frozen Glucose.

- Bromocresol purple, an indicator dye was added for colour change.

- The same procedure was followed for other sugars like Arabinose, Sucrose, Mannitol, Mannose, Lactose, Melibiose and Maltose.

- No colour change confirms presence of *P. gingivalis*.

- If the colour changes to yellow, it indicates *Prevotella intermedia*.

- Principle: Different microbial species metabolize sugars through different metabolic pathways depending on aerobic or anaerobic environment. Fermenting bacteria when grown in a liquid culture medium containing the
carbohydrate, produce organic acids as by-products. Due to these acidic by-products, the pH of medium lowers. If a pH indicator such as phenol red or bromocresol blue or purple is added to the medium, the acid production will change the medium from its original color to yellow. If no change observed in the colour of medium then sugar is not degraded by the organism.

- Based on the differences in these metabolic sugar fermentation by-products; *Porphyromonas gingivalis* was identified.

- The different sugar fermentation tests performed are mentioned below:

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Sugars</th>
<th>Colour change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose</td>
<td>Purple</td>
</tr>
<tr>
<td>2</td>
<td>Arabinose</td>
<td>Purple</td>
</tr>
<tr>
<td>3</td>
<td>Sucrose</td>
<td>Purple</td>
</tr>
<tr>
<td>4</td>
<td>Mannitol</td>
<td>Purple</td>
</tr>
<tr>
<td>5</td>
<td>Mannose</td>
<td>Purple</td>
</tr>
<tr>
<td>6</td>
<td>Lactose</td>
<td>Purple</td>
</tr>
<tr>
<td>7</td>
<td>Melibiose</td>
<td>Purple</td>
</tr>
<tr>
<td>8</td>
<td>Maltose</td>
<td>Purple</td>
</tr>
</tbody>
</table>

- Sugar fermentation tests were done to differentiate between *Porphyromonas gingivalis* and *Prevotella intermedia* (Photograph 21)
Step IV

**Antimicrobial activity assessed using ex-vivo model** (Photograph 22):

- **Procurement of extracted teeth and their preparation**
  1. A sufficient number of extracted, multi-rooted human deciduous teeth, including primary maxillary and mandibular molars with almost 2/3\textsuperscript{rd} of the roots present were selected for the study.
  2. The teeth were cleaned with periodontal curettes to remove periodontal tissues and bone and stored in saline.
  3. The teeth were then sterilized in autoclave at 121 °C and 15 psi for 20 minutes.
  4. Access cavity preparation was done using sterile burs with air-rotor under continuous water cooling.
  5. After access cavity preparation the extracted deciduous molars were again stored in saline and were subjected to sterilization by autoclave prior to their use in the ex-vivo model.

- **Ex-vivo Model for antimicrobial activity of various combinations of intracanal medicaments and vehicles on selected pathogens cultured from deciduous molars with necrotic pulp**
  1. The identified colonies of microorganisms namely *Streptococcus* *spp* and *Enterococcus faecalis* were then picked up by means of a loop, grown in broth and were adjusted to 0.5 McFarland standard. *Porphyromonas gingivalis* inoculum was adjusted to 1.0 McFarland standard.
  2. *Streptococcus* *spp*, and *Porphyromonas gingivalis* was lawn cultured on Blood agar whereas *Enterococcus faecalis* was on Brain heart infusion agar.
3. After the agar plates were made, checked for sterility and inoculated in vertical laminar airflow unit, the sterilized teeth were placed in the agar with a pair of sterile forceps.

**Preparation of the 2Mix:**

For the Ciprofloxacin, Metronidazole and Amoxicillin clavulanate tablet, the enteric coating is removed with a scalpel. The tablet is pulverized using mortar and pestle and, the powder is segregated. Antibiotics are prepared freshly before every use. Antibiotic paste remains are discarded and single antibiotic powders are stored sealed in air tight containers separately. The 2 antibiotics are mixed together with respective vehicles in a ratio of 1:1:1. A creamy consistency is prepared with good handling characteristics. Then this mix is used wherever essential.\(^{56,58,59}\)

**Gel preparation:**

To make the handling characteristics similar to the DAP and modified DAP antibiotic pastes, instead of using 2% CHX which was available commercially as irrigating solution, 2% CHX gel was formulated. The following chemicals have been procured from various suppliers for the preparation of 2% Chlorhexidine gel:

1. Hydroxypropyl methylcellulose (HPMC) – IP grade
2. Distilled water
3. 20% Chlorhexidine Gluconate – IP Grade

**Procedure for 2% Chlorhexidine gel preparation:**

The required quantity of HPMC were measured using electronic weighing balance. Distilled water and Chlorhexidine gluconate was measured using measuring beaker. Initially 4 % w/v HPMC gel was prepared by taking two thirds of the total
amount of freshly prepared distilled water. Then 4 gms of polymer (HPMC) was dissolved slowly by adding small increments to disperse with continuous stirring on a magnetic stirrer. The magnetic stirrer was kept at a low speed at 300- 400 rpm in order to avoid frothing. After incorporation of the premeasured polymer was complete, 10 ml of 20% Chlorhexidine Gluconate solution was added slowly i.e., dropwise to this HPMC gel with continuous stirring on a magnetic stirrer for geometric and uniform dispersion. Finally, cold distilled water was added to make the gel up to weight. The 2% Chlorhexidine gel thus obtained was triturated to a uniform consistency and left overnight to equilibrate.\textsuperscript{132,133}

**Preparation of DAP:** Ciprofloxacin, and Metronidazole was taken in a ratio of 1:1.

**Preparation of Modified DAP:** Amoxicillin clavulanate, and Metronidazole was taken in a ratio of 1:1.

**Preparation of Chlorhexidine gluconate:** 2% Chlorhexidine gluconate will be taken in one increment using a standard plastic filling or cement carrier. It will be taken in the ratio of 1:1 by volume with respect to the vehicle. These medicaments were placed on the floor of the pulp chamber of the deciduous molars and a temporary restoration (CAVIT) was placed to seal the acesss opening in the teeth.

Zones of inhibition were noted after 24 hrs for *Streptococcus spp* whereas 48 hrs for *Enterococcus faecalis* and *Porphyromonas gingivalis*. The following zones of inhibition of the various combinations were recorded for:

- **Group A:** Ciprofloxacin and Metronidazole (Double antibiotic paste) with glycerine in the ratio of 1:1 by volume,
- **Group B:** Ciprofloxacin and Metronidazole (Double antibiotic paste) with polyethylene glycol in the ratio of 1:1 by volume.
Materials & Methods

- Group C: Ciprofloxacin and Metronidazole (Double Antibiotic Paste-DAP) with propylene glycol in the ratio of 1:1 by volume,
- Group D: Amoxicillin clavulanate plus Metronidazole (Modified Double Antibiotic Paste) with glycerine in the ratio of 1:1 by volume.
- Group E: Amoxicillin with clavulanate plus Metronidazole (Modified Double Antibiotic Paste) with polyethylene glycol in the ratio of 1:1 by volume.
- Group F: Amoxicillin with clavulanate plus Metronidazole (Modified Double Antibiotic Paste) with propylene glycol in the ratio of 1:1 by volume.
- Group G: 2% Chlorhexidine gluconate gel with glycerine in the ratio of 1:1 by volume.
- Group H: 2% Chlorhexidine gluconate gel with polyethylene glycol in the ratio of 1:1 by volume and
- Group I: 2% Chlorhexidine gluconate gel with propylene glycol in the ratio of 1:1 by volume.

These zones of inhibitions were measured with the help of a measuring scale and entered in an Excel sheet. The data was entered, coded and recoded by the principal investigator under the guidance of an experienced statistician and epidemiologist prior to entry in the computer.

B. Secondary Objective (Polymerase Chain Reaction):

Step V:
Lab Protocol for Polymerase chain reaction

1. Isolation of Genomic DNA
2. Quantification of DNA by Bio photometer
3. Polymerase chain reaction
4. Agarose Gel Electrophoresis or Gel Documentation
Materials & Methods

1. **Step-wise Isolation of Genomic DNA from biomaterial obtained from infected root canals of teeth**

1. Collect the endodontic sample in a eppendorf containing Reduced Transport Fluid (RTF).
2. Centrifuge at 12,500 rpm for 5 min.
3. Add lysozyme 2 µl and keep for 10 mins.
4. Discard the supernatant. Add fresh 200 µl fresh T.E. buffer centrifuge for 3-4 minutes.
5. Repeat above procedure for 3-4 times with fresh T.E. buffer.
6. Discard supernatant add 50 µl lysis buffer I, keep it for 3 mins.
7. Add 50 µl lysis buffer II, keep it for 3 mins and 10 µl Proteinase – K(10mg/ml).
   Keep it in water bath at 60 °C for 2 hrs then keep in boiling water bath for 10 minutes.
8. Centrifuge the sample at 8000 rpm for 3 mins. Collect the 100 µl supernatant in fresh tube.
9. Add 10 µl of 3 M Sodium acetate + 300 µl of Absolute ethanol and keep the eppendorf tubes overnight at -20 °C.
10. Centrifuge at 12,000 rpm for 5 min. Discard the supernatant.
11. Add 100 µl of 70% Ethanol and centrifuge at 10,000 rpm for 5 mins. Discard the supernatant.
12. Add 100 µl of TE buffer and vortex for 2-3 mins.
13. Store the extracted DNA from endodontic biomaterial sample at -20 °C.129,130
2. **Quantification of DNA by Bio photometer**

1. Dilute the stock DNA appropriately using Molecular grade water. Dilution done in the ratio of 2:98 i.e., 2 µl of DNA + 98 µl of Molecular grade water.
2. Measure absorbance (OD) at the wavelengths 260 and 280 nm using UV spectrophotometer.
3. OD of 1 at 260 nm is equivalent to a standard value 50 µg/ml.
4. Final concentration of DNA= OD at 260 nm X Dilution factor X 50.
5. The ratio A260/A280 of 1.6-1.8 is indicative of good purity.\(^{129,130}\)

**Step VI:**

3. **Polymerase chain reaction (PCR)**

- Conventional PCR assay for *E. faecalis\(^{135}\):*
  1) The primer sequences for *E. faecalis* were designed based on literature from Sedgley et al, 2005.\(^{135}\) The primers specificity was confirmed by National Centre for Biotechnology Information (NCBI) by BLAST analysis.
  2) Following is a list of the PCR primers used in the current study for *Enterococcus faecalis:*

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Primer (5’to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CCGAGTGCTTGCACTCAATTGG</td>
</tr>
<tr>
<td>Reverse</td>
<td>CTCTTATGCCATGCGGCATAAAC</td>
</tr>
</tbody>
</table>
3) To determine temperature for optimal primer annealing for PCR specificity experiment, temperature gradient PCR assays were performed, using positive control DNA from *E. faecalis* ATCC 35550 and molecular grade water as negative control in the Veriti 96-Well Fast Thermal cycler (Applied Biosystems NY).

4) 100 ng total DNA template was prepared for 25 µl PCR amplification. 2X Master Mix (Amplicon) containing 2.5 U of red Taq DNA polymerase was used as per manufacturer’s instructions. 7.5 pmole of each primer was added to the reaction mixture.

5) The PCR amplification protocol was as follows: -

Five minutes DNA denaturing step at 95 °C was followed by 35 consecutives cycles at 94 °C for 30 secs; 60 °C for 45 secs and 72 °C for 15 secs. Following is the tabular representation:

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95 °C</td>
<td>5 minute</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>30 secs</td>
<td>35 cycles</td>
</tr>
<tr>
<td>Annealing</td>
<td>60 °C</td>
<td>45 secs</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>15 secs</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 °C</td>
<td>10 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Soak</td>
<td>4 °C</td>
<td>Indefinite</td>
<td></td>
</tr>
</tbody>
</table>

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81
PCR assay for Multiplex of *P. gingivalis* and *Treponema denticola*:

1) The multiplex PCR was performed by using specific primers for the 16S rRNA gene of each bacterium.

2) Following is a list of the PCR primers used in the current study for *P. gingivalis* and *Treponema denticola*:

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. gingivalis</em></td>
<td>GG CAG CTT GCC ATA CTG CG</td>
</tr>
<tr>
<td>Forward</td>
<td>ACT GTT AGC AAC TAC CGA TGT</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
</tr>
<tr>
<td><em>Treponema denticola</em></td>
<td>TAA TAC CGA ATG TGC TCA TTT ACA T</td>
</tr>
<tr>
<td>Forward</td>
<td>TCA AAG AAG CAT TCC CTC TTC TTA</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
</tr>
</tbody>
</table>

3) PCR amplification reactions were carried out in a reaction mixture in a final volume of 25 μl consisting of 3 μl of DNA sample, and 22 μl of reaction mixture containing 7.5 pmol of each primer, 200 μM of a mixture of deoxynucleoside triphosphates, 1.5 mM MgCl₂, 10X PCR buffer (10 mM Tris-HCl, pH 8.0), 50 mM KCl, 2.5 U RED Taq DNA Polymerase.

4) The PCR amplification protocol was as follows:

- 95 °C for 5 min followed by 40 cycles of 95 °C for 30 secs, 60 °C for 1 min, 72 °C for 1 min, and a final step of 72 °C for 10 min. Following is the tabular representation:

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95 °C</td>
<td>5 minute</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>30 secs</td>
<td>40 cycles</td>
</tr>
<tr>
<td>Annealing</td>
<td>60 °C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 °C</td>
<td>10 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Soak</td>
<td>4 °C</td>
<td>Indefinite</td>
<td></td>
</tr>
</tbody>
</table>
4. **Agarose Gel Electrophoresis to visualize isolated DNA**

   The PCR amplicons were analyzed by electrophoresis on a 2% agarose gel and stained with 0.5 µg/ml ethidium bromide.

**Procedure**

1. Weigh about 1 gm of agarose into a conical flask. Add 50 ml of 1xTAE
2. Boil for about 1 minute to completely dissolve the agarose.
3. Keep it to bench cool for 5 minutes to about 60 °C just to be hot enough to be held by bare hands.
4. Add 0.5µg/ml of ethidium bromide and mix well but with caution.
5. To cast the gel, pour the gel slowly in to the gel casting tray to avoid the entrapment of bubbles.
6. Insert the comb and check the comb is positioned accurately.
7. Allow it to set adequately for about 30 mins to 1 hour, with the lid on the tank.
8. The running buffer i.e., 1xTAE is poured into the gel tank to submerge the gel to 2-5 mm depth.\(^{129,130}\)

**Running of Agarose Gel**

1) Mix well by pipetting, 5ul of the loading dye with 10-20ul of PCR products and load in the wells carefully; along with respective molecular weight markers.
2) Apply the current with fixed voltage (80-100V).
3) The distance DNA has migrated was judged by migration of tracking dyes.
4) After electrophoresis DNA can be visualize by placing the gel in transilluminator (gel documentation) (Syngene, UK).
5) Linear DNA migrates through agarose gel with a mobility that is inversely proportional to the log10 of their molecular weight i.e., heavier the DNA in terms of its molecular weight, slower it travels through the gel.

6) Hence, different concentration of agarose affect the porosity of the gel and can resolve different sizes of DNA fragments.\textsuperscript{129,130}

GEL DOCUMENTATION\textsuperscript{129,130}:

Procedure

1) PCR products were visualised under ultraviolet light using Syngene gel documentation. A 100-bp DNA ladder served as the molecular weight marker.

2) The identity of each band was concluded by visual comparison with a molecular weight ladder. Using a 302nm UV transilluminator and orange emission filter, images of ethidium bromide stained DNA gels were captured in a fraction of second.

3) Gel documentation of PCR generated DNA bands of \textit{Enterococcus faecalis} positive samples following standardization by electrophoresis are confirmed (Photograph 23).

4) Gel documentation of PCR generated DNA bands of \textit{P. gingivalis} and \textit{Treponema denticola} samples following standardization by electrophoresis are confirmed (Photograph 24).
Outcome Measures:

1. Comparison of all 9 medicament groups for each organism isolated, namely *Streptococcus* spp, *Porphyromonas gingivalis* and *Enterococcus faecalis*.

2. Comparison of the medicaments with the same vehicle against the above mentioned organisms.

3. Comparison of the vehicles with the same medicament against the above mentioned organisms.

4. To identify and compare selected microorganisms from root canals of infected deciduous molars through microbial culture and PCR.
Data analysis plan:

The data was entered in the excel sheet for statistical analysis. Statistical analysis was done using SPSS software version 22. Mean, Median and Standard Deviation of the data was assessed wherever necessary.

Phase 1:

*In vitro study*

- Mean Value and SD
- Kruskal Wallis test
- Pairwise comparison using post hoc Mann Whitney U test (p<0.05).

Phase 2:

*Main study*

- Tests of Normality by Shapiro-Wilk tests.
- Kruskal Wallis test– Intergroup comparison
- Post-hoc comparison by Mann-Whitney U test.
- Statistical Difference in detection of *P. gingivalis* and *E. faecalis* by culture and PCR by McNemar test.

Level of significance was set at p<0.5 for all the above tests applied.

We also compared the results using parametric tests and results were the same except for the difference between vehicles which was statistically significant only for Modified DAP combined with glycerine and propylene glycol. Though the data is Quantitative on applying Tests of Normality and even after log transformation, the distribution was not normal, hence Non-parametric tests were applied and analysed.
Phase 1A: *In vitro* Determination of Bactericidal activity of vehicles against ATCC strains by broth dilution

**Photograph 1:** Susceptibility of *Streptococcus mutans* ATCC 25175 to (A) Propylene glycol, (B) Glycerine, (C) PEG 400, (D) PEG 1000 and (E) Propylene glycol with PEG 400 assessed using Broth dilution assay.
Phase 1A: *In vitro* Determination of Bactericidal activity of vehicles against ATCC strains by broth dilution

Photograph 2: Minimum Bactericidal Concentration (MBC) of *Streptococcus mutans* ATCC 25175 to (A) Propylene glycol, (B) Glycerine, (C) PEG 400, (D) PEG 1000 and (E) Propylene glycol with PEG 400 was assessed after being subcultured on BHI agar.
Phase 1 B: *In vitro* Antibacterial activity of endodontic medicaments and vehicle combinations against ATCC strains by agar well diffusion method using pure drugs

**Photographs 3:** MIC of single antimicrobial substances i.e., (A) Chlorhexidine gluconate (B) Ciprofloxacin (C) Amoxicillin clavulanate and; (D) Metronidazole by broth dilution method against *P. gingivalis*.

**Photographs 4:** MIC of combination of antimicrobial substances i.e., (A) Ciprofloxacin with Metronidazole and; (B) Amoxicillin clavulanate with Metronidazole by broth dilution method against *P. gingivalis*. 
Phase 1 B: *In vitro* Antibacterial activity of endodontic medicaments and vehicle combinations against ATCC strains by agar well diffusion method using pure drugs

Photographs 5: Antimicrobial activity of the endodontic medicaments i.e., (A) 2% Chlorhexidine gluconate (B) Ciprofloxacin with Metronidazole and; (C) Amoxycillin clavulanate with Metronidazole along with the four vehicle combinations against *P. gingivalis* using agar well diffusion method.
Phase 1 C: *In vitro* Antibacterial activity of innovative endodontic medicaments and different vehicle combinations against ATCC strains by agar well diffusion method using commercial drugs.

**Photograph 6:**
Antibacterial activity of Ciprofloxacin with Metronidazole (C+M) against
(A) *Streptococcus mutans*
(B) *Staphylococcus aureus*
(C) *Enterococcus faecalis* and
(D) *Eschericia coli*

**Photograph 7:**
Antibacterial activity of Amoxicillin clavulanate with Metronidazole (A+M) against
(A) *Streptococcus mutans*
(B) *Staphylococcus aureus*
(C) *Enterococcus faecalis* and
(D) *Eschericia coli*

**Photograph 8:**
Antibacterial activity of 2% Chlorhexidine (CHX) against
(A) *Streptococcus mutans*
(B) *Staphylococcus aureus*
(C) *Enterococcus faecalis* and
(D) *Eschericia coli*
Phase 2 A: Primary objective - Antibacterial activity of various combination of intracanal medicaments and vehicle

Photograph 9: Intra-oral view showing abscess with 85

Photograph 10: Rubber dam isolation of the tooth with radiograph (inset) showing radiolucency in furcation area

Photograph 11: Clinical and radiographic working length prior to endodontic sample collection from each root canal with # 15 K file.

Photograph 12: Absorbent paper points placed in the canals for collection of endodontic sample

Photograph 13: Eppendorf containing # 15 K file and 2 paper points from each root canal respectively.
Photograph 14: Cultured petri plates with endodontic sample incubated in CO$_2$ jar

Photograph 15: Cultured petri plates with endodontic sample incubated in anaerobic jar

Photograph 16: Blood agar plate after incubation in CO$_2$ jar

Photograph 17: Blood agar plate after incubation in anaerobic jar

Photograph 18: Enterococcus confirmatory agar after incubation in CO$_2$ jar

Photograph 19: Kanamycin blood agar plate after incubation in anaerobic jar
Photograph 20: Confirmation of isolated organisms under stereo microscope after Gram staining

(A) Streptococcus spp.
(B) Porphyromonas gingivalis
(C) Enterococcus faecalis

Photograph 21: Confirmation of Porphyromonas gingivalis by sugar test i.e. glucose arabinose, sucrose, mannitol, mannose, lactose, melibiose, maltose and control. No colour change (purple) confirms presence of P. gingivalis
Photograph 22: Evaluation of antibacterial activity of innovative endodontic medicaments and different vehicle combinations using *ex-vivo* method against

(A) Clinically isolated *Streptococcus* spp.

(B) Clinically isolated *Porphyromonas gingivalis*

(C) Clinically isolated *Enterococcus faecalis*
Phase 2 B: Secondary objective – Polymerase Chain Reaction

Photograph 23: Conventional PCR of *E. faecalis* followed by agarose gel documentation of DNA bands generated by electrophoresis

Photograph 24: Multiplex PCR of *Porphyromonas gingivalis* and *Treponema denticola* followed by agarose gel documentation of DNA bands generated by electrophoresis
RESULTS

Phase 1:

A. *In vitro* Determination of Bactericidal activity of vehicles against ATCC strains by broth dilution

The results for the Minimum Bactericidal activity of the vehicles PG, Glycerine, PEG 400, PEG 1000 and PG+PEG 400 are depicted in Table 1. All vehicles exhibited bactericidal activity though at different concentrations on the ATCC strains of *Staphylococcus aureus*, *S. mutans*, *E. faecalis* and *Escherchia coli* (*E. coli*). Propylene glycol exhibited bactericidal activity at 50% against *S. mutans*, 100% against *Staphylococcus aureus*, 25% against *E. faecalis* and 50% against *E. coli*. Glycerine, PEG 400 and PG + PEG 400 exhibited bactericidal activity at only 100% against all the organisms making them the least bactericidal vehicle amongst above mentioned vehicles. PEG 1000 was bactericidal against *S. mutans* and *E. coli* at 25%, while against *Staphylococcus aureus* and *E. faecalis* at 100%.

Of all the ATCC strains of microorganisms, *S. mutans* and *E. coli* were the most susceptible to the vehicles, *E. faecalis* exhibited intermediate susceptibility and *S. aureus* was the most resistant to all the vehicles. Propylene glycol showed bactericidal activity against maximum number of organisms i.e., three of which *E. faecalis*, commonly associated with root canal treatment failure was terminated at a very low concentration of 25 %. PEG 1000 exhibited bactericidal activity against *S. mutans* and *E. coli* at the lowest concentration and maximum dilution i.e., 25 %. Glycerine and combination of Propylene glycol and PEG 400 were the vehicles with least bactericidal activity against selected pathogens. Combination of Propylene glycol and PEG 400 did not show any synergistic antimicrobial activity and infact its
efficacy decreased against *S. mutans*, *E. faecalis* and *E. coli* in comparison to Propylene glycol alone.

**B. In vitro Antibacterial activity of endodontic medicaments and vehicle combinations against ATCC strains by agar well diffusion method using pure drugs**

Initially MIC was evaluated for single endodontic medicaments only and all organisms were resistant to Metronidazole (*Table 2a*). MIC of Chlorhexidine against *S. mutans*, *S. aureus* and *E. coli* was 0.078% whereas against *E. faecalis* and *P. gingivalis* was 0.156% and 0.019% respectively. MIC of Ciprofloxacin against *S. mutans* was 7.81µg/ml, *S. aureus* was 31.25µg/ml, and *P. gingivalis* was 0.019µg/ml. MIC of Ciprofloxacin against *E. faecalis* and *E. coli* was 1.95µg/ml. MIC of Amoxycillin clavulanate against *S. mutans* was 7.8125µg/ml, *S. aureus* was 3.90µg/ml, and *P. gingivalis* was 0.019µg/ml. MIC of Amoxycillin clavulanate against *E. faecalis* and *E. coli* was 15.625µg/ml. All organisms exhibited resistance to Metronidazole when used alone as depicted by the extremely high MIC values.

Later MIC of combination of endodontic medicaments i.e., Ciprofloxacin with Metronidazole and Amoxicillin clavulanate with Metronidazole were carried out. Following combination of Ciprofloxacin with Metronidazole, MIC values of *S. mutans*, *S. aureus* and *P. gingivalis* further reduced while Amoxicillin clavulanate with Metronidazole combination reduced MIC values against *S. mutans* only (*Table 2b*). MIC of Ciprofloxacin with Metronidazole against *S. mutans*, *E. faecalis* and *E. coli* was 1.95µg/ml; where as against *S. aureus* and *P. gingivalis* was 7.81µg/ml and 0.039µg/ml respectively. MIC of Amoxicillin clavulanate with Metronidazole against *S. mutans* and *P. gingivalis* was 3.90µg/ml and 0.019µg/ml respectively. MIC of
Amoxicillin clavulanate with Metronidazole against *S. aureus*, *E. faecalis* and *E. coli* was 15.625 µg/ml.

**Table 2c: Antimicrobial activity of the endodontic medicaments and vehicle combinations against selected pathogens using agar well diffusion method**

The zones of inhibition of all of the selected organisms were recorded to measure their antibacterial activity using agar well diffusion method (Table 2c). Amongst the mean zones of inhibition against *S. mutans* using the CHX combination with the four vehicles, the highest was for CHX+PG (32.00±1.00) but the difference in comparison to CHX and other vehicles combination was not significant. Similarly, the highest zone of inhibition for A+M and the four vehicle combination against *S. mutans* was of A+M+PG (26.33±0.57) again. C+M with all the four vehicles against *S. mutans* exhibited resistance which is shown by the no zones of inhibition recorded in the triplicates performed.

Amongst the mean zones of inhibition against *S. aureus* using the CHX endodontic medicament combination with the four vehicles, the highest was for CHX+PEG (39.00±1.00) but the difference between CHX and other vehicles was not significant. The highest zone of inhibition amongst C+M and the four vehicle combination against *S. aureus* was of C+M+PEG (25.00±1.00) again. Similarly, the highest zone of inhibition for A+M and the four vehicle combination against *S. aureus* was of A+M+PG and A+M+PEG (29.00±1.00). The difference amongst all four vehicle combinations was not statistically significant.

Amongst the mean zones of inhibition against *E. faecalis* using the CHX endodontic medicament combination with the four vehicles, the highest was for CHX+PG (31.67±1.52) but the difference between CHX and other vehicles was not significant. The highest zone of inhibition amongst C+M and the four vehicle
combination against *E. faecalis* was C+M+PEG (34.00±1.00). Similarly, the highest zone of inhibition for A+M and the four vehicle combination against *E. faecalis* was of A+M+Glycerine (28.00±1.00). The difference amongst all four vehicle combinations was not statistically significant.

Amongst the mean zones of inhibition against *P. gingivalis* using the CHX endodontic medicament combination with the four vehicles, the highest was for CHX+Glycerine (27.67±1.52) but the difference between CHX and other vehicles was not significant. The highest zone of inhibition amongst C+M and the four vehicle combination against *P. gingivalis* was C+M+PG+PEG (39.00±1.00)). Similarly, the highest zone of inhibition for A+M and the four vehicle combination against *P. gingivalis* was of A+M+PG (46.33±1.52). There was no statistically significant difference in the same antimicrobial drug and vehicles i.e. PEG, PG, PEG + PG and Glycerine except *P. gingivalis*. There existed significant difference in C+M+PG and C+M+ Glycerine only on *P. gingivalis*.

All the organisms tested were more susceptible to one of the endodontic medicament and vehicle combinations but the difference was not statistically significant except for *P. gingivalis*. *S. mutans* and *S. aureus* were more susceptible to Chlorhexidine than C+M and A+M as endodontic medicaments which is shown by the larger zones of inhibition. *E. faecalis* was most susceptible to C+M followed by Chlorhexidine and lastly A+M. Also, A+M was most effective against *P. gingivalis*, followed by C+M and lastly, Chlorhexidine. The zones of inhibition of *P. gingivalis* were larger in comparison to other facultative anaerobes, which shows that the obligate anaerobes are more susceptible.
C. \textit{In vitro} Antibacterial activity of innovative endodontic medicaments and different vehicle combinations against ATCC strains by agar well diffusion method using commercial drugs

Table 3: \textit{In vitro} Antibacterial activity of innovative endodontic medicaments and different vehicle combinations against ATCC strains by agar well diffusion method using commercial drugs

The zones of inhibition against \textit{S. mutans}, \textit{S. aureus}, \textit{E. faecalis} and \textit{E. coli} were recorded using commercial drugs for preparing the combinations of the intracanal medicaments and vehicles to measure their antibacterial activity by agar well diffusion method (Table 3).

Amongst the mean zones of inhibition against \textit{S. mutans} using the 2% CHX combination with the four vehicles, the highest was for CHX+PEG+PG (20.67±0.577) followed by CHX+PEG (20.33±0.577) and lastly CHX+Glycerine and CHX+PG were (20.00±0.000). The highest zone of inhibition for C+M and the four vehicle combination against \textit{S. mutans} was of C+M+Glycerine (22.33±0.577). A+M with all the four vehicles against \textit{S. mutans} exhibited maximum zone of inhibition with A+M+PG (33.00±0.000).

Amongst the mean zones of inhibition against \textit{S. aureus} using the CHX endodontic medicament combination with the four vehicles, the highest was for CHX+PEG (21.33±0.577). The highest zone of inhibition amongst C+M and the four vehicle combination against \textit{S. aureus} was of C+M+Glycerine (23.33±0.577). Similarly, the highest zone of inhibition for A+M and the four vehicle combination against \textit{S. aureus} was of A+M+Glycerine (33.67±0.577).

Amongst the mean zones of inhibition against \textit{E. faecalis} using the CHX endodontic medicament combination with the four vehicles, the highest was for
CHX+PEG+PG (21.67±0.577). The highest zone of inhibition amongst C+M and the four vehicle combination against *E. faecalis* was C+M+Glycerine (26.00±1.00) and C+M+PG+PEG (26.00±1.000). Similarly, the highest zone of inhibition for A+M and the four vehicle combination against *E. faecalis* was of A+M+PG (31.33±0.577).

Lastly, *E. coli* was tested as a control as per CLSI guidelines for antimicrobial susceptibility testing while *P. gingivalis* being susceptible to the drug and vehicle combinations was not selected. Amongst the mean zones of inhibition against *E. coli* using the CHX endodontic medicament combination with the four vehicles, the highest was for CHX+PG+PEG (21.33±0.577). The highest zone of inhibition amongst C+M and the four vehicle combination against *E. coli* was of C+M+PG and C+M+PEG (25.00±0.000). Similarly, the highest zone of inhibition for A+M and the four vehicle combination against *E. coli* was of A+M+PG (32.33±1.528).

Overall observations can be summarized as the most effective medicament-vehicle combination effective against *S. mutans* is A+M+PG, for *S. aureus* is A+M+Glycerine, for *E. faecalis* is A+M+PG and *E. coli* is A+M+PG closely followed by C+M+PG or PEG. Chlorhexidine is the least effective while A+M is the most effective intracanal medicament. Of all the vehicles, PG is the most effective vehicle when used in combination with intracanal medicaments.

**Phase 2:**

The distribution of males was higher (57 %) in comparison to the female children (43 %) who participated in the study on basis of inclusion and exclusion criteria. The deciduous mandibular molar teeth showed a higher distribution of necrotic pulp (75 %) as compared to deciduous maxillary molars (25 %). *(Pie chart 1).*
A. **Primary Objective-Antimicrobial activity of various combinations of intracanal medicaments and vehicles**

**Table 4 : Organisms detected and their association with signs and symptoms present in subjects**

There was no significant relation between any of the organisms detected and the signs and symptoms present including radiolucency detected in furcation or periapical area radiographically. Though the association was not statistically significant, the following are the highest percentages of detection of organism combinations in relation to each sign and symptom recorded.

History of pain was detected in 92.3 % of root canals detecting *Streptococcus spp* and *E. faecalis*, tenderness to percussion was detected in 32.7 % of root canals detecting *Streptococcus spp* and *P. gingivalis*. Swelling was detected in 66.7 % of root canals detecting all the selected microorganisms, followed by 55.8 % in root canals detecting *Streptococcus spp* and *P. gingivalis* and 23.1 % with root canals detecting *Streptococcus spp* and *E. faecalis*. Teeth with draining sinus had the highest percentage of 16.7 % in root canals detecting all the selected microorganisms. Vital root canals have lesser microbial load (27.7 %) as compared to non-vital root canals (72.3 %). Lastly, as only teeth diagnosed as necrotic due to the presence of periradicular radiolucency were included in the study; association of organisms detected with it and its statistical significance could not be estimated; as there exists no comparison for the teeth with periradicular radiolucency.
Table 5a (Diagram 1a): Comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicle combinations against *Streptococcus spp*

The mean zone of inhibition (mms) against *Streptococcus spp* of the C+M+G group was 28.75 ± 7.11, A+M+G was 31.32 ± 6.85, CHX+G was 23.21 ± 6.29, C+M+PEG was 26.03 ± 8.36, A+M+PEG was 31.95 ± 7.13, CHX+PEG was 23.84 ± 7.52, C+M+PG was 28.60 ± 7.82, A+M+PG was 32.26 ± 7.77 and CHX+PG was 22.91 ± 7.71. There was statistically significant difference (p<0.001) between the nine groups i.e. C+M+G, A+M+G, CHX+G, C+M+PEG, A+M+PEG, CHX+PEG, C+M+PG, A+M+PG and CHX+PG against *Streptococcus spp*.

Table 5b (Diagram 1b): Comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicles combinations against *Porphyromonas gingivalis*

The mean zone of inhibition (mms) against *Porphyromonas gingivalis* of the C+M+G group was 29.67 ± 8.41, A+M+G was 35.75 ± 9.28, CHX+G was 21.15 ± 6.21, C+M+PEG was 29.75 ± 7.39, A+M+PEG was 35.25 ± 7.61, CHX+PEG was 22.74 ± 5.79, C+M+PG was 30.42 ± 7.01, A+M+PG was 39.37 ± 9.27 and CHX+PG was 20.67 ± 5.41. There was statistically significant difference (p<0.001) between the nine groups i.e. C+M+G, A+M+G, CHX+G, C+M+PEG, A+M+PEG, CHX+PEG, C+M+PG, A+M+PG and CHX+PG against *Porphyromonas gingivalis*.

Table 5c (Diagram 1c): Comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicles combinations against *Enterococcus faecalis*

The mean zone of inhibition (mms) against *Enterococcus faecalis* of the C+M+G group was 30.21 ± 7.25, A+M+G was 33.58 ± 7.24, CHX+G was 26.58 ±
6.31, C+M+PEG was 28.47 ± 6.96, A+M+PEG was 34.53 ± 7.34, CHX+PEG was 25.68 ± 7.17, C+M+PG was 31.11 ± 10.66, A+M+PG was 33.79 ± 8.54 and CHX+PG was 25.16 ± 8.46. There was statistically significant difference (p<0.001) between the nine groups i.e. C+M+G, A+M+G, CHX+G, C+M+PEG, A+M+PEG, CHX+PEG, C+M+PG, A+M+PG and CHX+PG against Enterococcus faecalis.

To know exactly which intracanal medicament and vehicle were effective, comparisons were done between all three medicaments keeping vehicles constant and all three vehicles keeping medicaments constant. This would give the idea of the effectiveness of each medicament and vehicle individually and which of the two to combine for maximum bactericidal effect and thus, effective disinfection of the root canals of deciduous necrotic molars.

Table 6a (Diagram 2a): Comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each vehicle against Streptococcus spp

The mean zones of inhibition in mms of the three medicaments with glycerine as vehicle against Streptococcus spp were C+M+G was 28.75 ± 7.11, A+M+G was 31.32 ± 6.85, CHX+G was 23.21 ± 6.29 and the difference between them was statistically significant (p<0.001). The mean zones of inhibition in mm sof the three medicaments with PEG as vehicle against Streptococcus spp were C+M+PEG was 26.03 ± 8.36, A+M+PEG was 31.95 ± 7.13, CHX+PEG was 23.84 ± 7.52 and the difference between them was statistically significant (p<0.001). The mean zones of inhibition in mm of the three medicaments with PG as vehicle against Streptococcus spp were C+M+PG was 28.60 ± 7.82, A+M+PG was 32.26 ± 7.77, CHX+PG was 22.91 ± 7.71 and the difference between them was statistically significant (p<0.001). The post-hoc comparison between groups having significant differences is presented in Table 6d.
Table 6b (Diagram 2b): Comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each vehicle against *P. gingivalis*

The mean zones of inhibition in mms of the three medicaments with glycerine as vehicle against *P. gingivalis* were C+M+G was 29.67 ± 8.41, A+M+G was 35.75 ± 9.28, CHX+G was 21.15 ± 6.21 and the difference between them was statistically significant (p<0.001). The mean zones of inhibition in mms of the three medicaments with PEG as vehicle against *P. gingivalis* were C+M+PEG was 29.75 ± 7.39, A+M+PEG was 35.25 ± 7.61, CHX+PEG was 22.74 ± 5.79 and the difference between them was statistically significant (p<0.001). The mean zones of inhibition in mms of the three medicaments with PG as vehicle against *P. gingivalis* were C+M+PG was 30.42 ± 7.01, A+M+PG was 39.37 ± 9.27, CHX+PG was 20.67 ± 5.41 and the difference between them was statistically significant (p<0.001). The post-hoc comparison between groups having significant differences is presented in Table 6d.

Table 6c (Diagram 2c): Comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each vehicle against *E. faecalis*

The mean zones of inhibition in mms of the three medicaments with glycerine as vehicle against *E. faecalis* were C+M+G was 30.21 ± 7.25, A+M+G was 33.58 ± 7.24, CHX+G was 26.58 ± 6.31 and the difference between them was statistically significant (p<0.001). The mean zones of inhibition in mms of the three medicaments with PEG as vehicle against *E. faecalis* were C+M+PEG was 28.47 ± 6.96, A+M+PEG was 34.53 ± 7.34, CHX+PEG was 25.68 ± 7.17 and the difference between them was statistically significant (p<0.001). The mean zones of inhibition in mms of the three medicaments with PG as vehicle against *E. faecalis* were C+M+PG was 31.11 ± 10.66, A+M+PG was 33.79 ± 8.54, CHX+PG was 25.16 ± 8.46 and the difference between them was statistically significant (p<0.001). The post-hoc
Results

Comparison between groups having significant differences is presented in Table 6d.

Table 6d: Post hoc comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each of the three vehicles against selected pathogens.

The difference between antimicrobial activity of all the three medicaments with each of the three vehicles against selected microorganisms was statistically significant.

Hence, post-hoc comparison between every two groups of medicament with the same vehicle against *Streptococcus spp* was done. For *Streptococcus spp* between every two groups of medicament with glycerine (G) as vehicle, A+M+G had greater antimicrobial activity as compared to C+M+G and the difference was statistically significant (p=0.009). C+M+G had greater antimicrobial activity than CHX+G and the difference was statistically significant (p<0.001). A+M+G had greater antimicrobial activity than CHX+G and the difference was statistically significant (p<0.001).

For *Streptococcus spp* between every two groups of medicament with PEG as vehicle, A+M+PEG had greater antimicrobial activity as compared to C+M+PEG and the difference was statistically significant (p=0.001). C+M+PEG had greater antimicrobial activity than CHX+PEG and the difference was statistically significant (p<0.001). A+M+PEG had greater antimicrobial activity than CHX+PEG and the difference was statistically significant (p<0.001).

For *Streptococcus spp* between every two groups of medicament with PG as vehicle, A+M+PG had greater antimicrobial activity as compared to C+M+PG and the difference was statistically significant (p=0.009). C+M+PG had greater antimicrobial activity than CHX+PG and the difference was statistically significant (p<0.001). A+M+PG had greater antimicrobial activity than CHX+PG and the
Results

difference was statistically significant (p<0.001).

Also, post-hoc comparison between every two groups of medicament with the same vehicle against *P. gingivalis* was done. For *P. gingivalis* between every two groups of medicament with glycerine(G) as vehicle, A+M+G had greater antimicrobial activity as compared to C+M+G and the difference was statistically significant (p=0.02). C+M+G had greater antimicrobial activity than CHX+G and the difference was statistically significant (p=0.001). A+M+G had greater antimicrobial activity than CHX+G and the difference was statistically significant (p<0.001).

For *P. gingivalis* between every two groups of medicament with PEG as vehicle, A+M+PEG had greater antimicrobial activity as compared to C+M+PEG and the difference was statistically significant (p=0.002). C+M+PEG had greater antimicrobial activity than CHX+PEG and the difference was statistically significant (p<0.001). A+M+PEG had greater antimicrobial activity than CHX+PEG and the difference was statistically significant (p<0.001).

For *P. gingivalis* between every two groups of medicament with PG as vehicle, A+M+PG had greater antimicrobial activity as compared to C+M+PG and the difference was statistically significant (p<0.001). C+M+PG had greater antimicrobial activity than CHX+PG and the difference was statistically significant (p<0.001). A+M+PG had greater antimicrobial activity than CHX+PG and the difference was statistically significant (p<0.001).

Also, post-hoc comparison between every two groups of medicament with the same vehicle against *E. faecalis* was done. For *E. faecalis* between every two groups of medicament with glycerine(G) as vehicle, A+M+G had greater antimicrobial activity as compared to C+M+G and the difference was not significant (p=0.15). C+M+G had greater antimicrobial activity than CHX+G and the difference was not
Results

significant (p=0.15). A+M+G had greater antimicrobial activity than CHX+G and the difference was statistically significant (p=0.005).

For *E. faecalis* between every two groups of medicament with PEG as vehicle, A+M+PEG had greater antimicrobial activity as compared to C+M+PEG and the difference was statistically significant (p=0.007). C+M+PEG had greater antimicrobial activity than CHX+PEG and the difference was not significant (p=0.24). A+M+PEG had greater antimicrobial activity than CHX+PEG and the difference was statistically significant (p<0.001).

For *E. faecalis* between every two groups of medicament with PG as vehicle, A+M+PG had greater antimicrobial activity as compared to C+M+PG and the difference was not significant (p=0.39). C+M+PG had greater antimicrobial activity than CHX+PG and the difference was statistically significant (p=0.03). A+M+PG had greater antimicrobial activity than CHX+PG and the difference was statistically significant (p=0.002).

**Table 7a (Diagram 3a):** Comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each medicament against *Streptococcus spp*

The mean zones of inhibition in mms of the C+M medicament with all the three vehicles against *Streptococcus spp* were C+M+G was 28.75 ± 7.11, C+M+PEG was 26.03 ± 8.36, C+M+PG was 28.60 ± 7.82 and the difference between them was statistically significant (p=0.033). The mean zones of inhibition in mms of the A+M medicament with with all the three vehicles against *Streptococcus spp* were, A+M+G was 31.32 ± 6.85, A+M+PEG was 31.95 ± 7.13, A+M+PG was 32.26 ± 7.77 and the difference between them was not significant (p=0.92). The mean zones of inhibition in mms of the CHX medicament with all the three vehicles against *Streptococcus spp* were, CHX+G was 23.21 ± 6.29, CHX+PEG was 23.84 ± 7.52,
Results

CHX+PG was 22.91 ± 7.71 and the difference between them was not significant (p=0.81). The post-hoc comparison between C+M medicament groups with all the three vehicles having significant differences in antimicrobial activity is presented in Table 7d.

**Table 7b (Diagram 3b):** Comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each medicament against *P. gingivalis*

The mean zones of inhibition in mms of the C+M medicament with all the three vehicles against *P. gingivalis* were C+M+G was 29.67 ± 8.41, C+M+PEG was 29.75 ± 7.39, C+M+PG was 30.42 ± 7.01 and the difference between them was not significant (p=0.93). The mean zones of inhibition in mms of the A+M medicament with all the three vehicles against *P. gingivalis* were A+M+G was 35.75 ± 9.28, A+M+PEG was 35.25 ± 7.61, A+M+PG was 39.37 ± 9.27 and the difference between them was statistically significant (p<0.034). The mean zones of inhibition in mms of the CHX medicament with all the three vehicles against *P. gingivalis* were CHX+G was 21.15 ± 6.21, CHX+PEG was 22.74 ± 5.79, CHX+PG was 20.67 ± 5.41 and the difference between them was not significant (p=0.20). The post-hoc comparison between A+M medicament groups with all the three vehicles having significant differences in antimicrobial activity is presented in Table 7d.

**Table 7c (Diagram 3c):** Comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each medicament against *E. faecalis*

The mean zones of inhibition in mms of the C+M medicament with all the three vehicles against *E. faecalis* were C+M+G was 30.21 ± 7.25, C+M+PEG was 28.47 ± 6.96, C+M+PG was 31.11 ± 10.66 and the difference between them was not significant (p=0.66). The mean zones of inhibition in mms of the A+M medicament with all the three vehicles against *E. faecalis* were A+M+G was 33.58 ± 7.24,
A+M+PEG was 34.53 ± 7.34, A+M+PG was 33.79 ± 8.54 and the difference between them was not significant (p=0.76). The mean zones of inhibition in mms of the CHX medicament with all the three vehicles against *E. faecalis* were, CHX+G was 26.58 ± 6.31, CHX+PEG was 25.68 ± 7.17, CHX+PG was 25.16 ± 8.46 and the difference between them was not significant (p=0.79).

**Table 7d: Post hoc comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each of the three medicaments against selected pathogens**

The difference between antimicrobial activity of all the three vehicles with each of the two medicaments i.e., C+M and A+M against *Streptococcus spp* and *Porphyromonas gingivalis* was statistically significant.

Hence, post-hoc comparison between the medicament i.e., C+M and with one of the three vehicles against *Streptococcus spp* was done. For *Streptococcus spp*, between the two groups of medicament with each of the three vehicles as carrier, C+M+G had greater antimicrobial activity as compared to C+M+PEG and the difference was statistically significant (p=0.02). C+M+G and C+M+PG had similar antimicrobial activity and the difference was not significant (p=0.87). C+M+PG had greater antimicrobial activity than C+M+PEG and the difference was statistically significant (p=0.04).

Therefore, of the three vehicles PG and G are conducive carriers for C+M against *Streptococcus spp* as antimicrobial activity is significantly better when combination is used.

Also, post-hoc comparison between the medicament i.e., A+M with one of the three vehicles against *P. gingivalis* was done. For *P. gingivalis*, between every two groups of medicament with each of the three vehicles as carrier, A+M+G had greater
Results

antimicrobial activity as compared to A+M+PEG and the difference was not significant (p=0.94). A+M+G had lesser antimicrobial activity to A+M+PG and the difference was not significant (p=0.06). A+M+PG had greater antimicrobial activity than A+M+PEG and the difference was statistically significant (p=0.009).

Therefore, of the three vehicles PG is a conducive carrier for A+M against *P. gingivalis* as antimicrobial activity is significantly better when combination is used.

B. Secondary Objective- PCR assay

- *Streptococcus spp* were detected in 100% of the endodontic samples from necrotic deciduous teeth by culture alone.
- *Treponema denticola* was detected in 63% of the endodontic samples from necrotic deciduous teeth by PCR alone.

Table 8a: Comparison of detection of *E. faecalis* by PCR and culture

*Enterococcus faecalis* was isolated in 18% of the endodontic samples from necrotic deciduous by culture and 41% by PCR and the difference in detection was statistically significant (p<0.001; McNemar test).

Table 8b: Comparison of detection of *P. gingivalis* by PCR and culture

*Porphyromonas gingivalis* was isolated in 58% of the endodontic samples from necrotic deciduous teeth by culture and 76% by PCR and the difference in detection was statistically significant (p<0.001; McNemar test).
Phase 1:

A. *In vitro* Determination of Bactericidal activity of vehicles against ATCC strains by broth dilution

Table 1: Bactericidal activity of vehicles against ATCC strains of *S. mutans*, *S. aureus*, *E. faecalis* and *E. coli*

<table>
<thead>
<tr>
<th>Vehicles</th>
<th><em>S. mutans</em></th>
<th><em>S. aureus</em></th>
<th><em>E. faecalis</em></th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Propylene glycol (PG)</td>
<td>50%</td>
<td>100%</td>
<td>25%</td>
<td>50%</td>
</tr>
<tr>
<td>Glycerine</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Polyethylene Glycol (PEG) 400</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Polyethylene Glycol (PEG) 1000</td>
<td>25%</td>
<td>100%</td>
<td>100%</td>
<td>25%</td>
</tr>
<tr>
<td>PG + PEG 400</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>
Phase 1:

B. *In vitro* Antibacterial activity of endodontic medicaments and vehicle combinations against ATCC strains by agar well diffusion method using pure drugs

**TABLE 2a : MIC of single antimicrobial substances by broth dilution method** ^5^

<table>
<thead>
<tr>
<th>Test Agent</th>
<th>Microorganism</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorhexidine</td>
<td><em>S. mutans</em></td>
<td>0.078%</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus</em></td>
<td>0.078%</td>
</tr>
<tr>
<td></td>
<td><em>E. faecalis</em></td>
<td>0.156%</td>
</tr>
<tr>
<td></td>
<td><em>P. gingivalis</em></td>
<td>0.019%</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td>0.078%</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td><em>S. mutans</em></td>
<td>7.81µg/ml</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus</em></td>
<td>31.25µg/ml</td>
</tr>
<tr>
<td></td>
<td><em>E. faecalis</em></td>
<td>1.95µg/ml</td>
</tr>
<tr>
<td></td>
<td><em>P. gingivalis</em></td>
<td>0.019µg/ml</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td>1.95µg/ml</td>
</tr>
<tr>
<td>Amoxicillin clavulanate</td>
<td><em>S. mutans</em></td>
<td>7.8125µg/ml</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus</em></td>
<td>3.90µg/ml</td>
</tr>
<tr>
<td></td>
<td><em>E. faecalis</em></td>
<td>15.625µg/ml</td>
</tr>
<tr>
<td></td>
<td><em>P. gingivalis</em></td>
<td>0.019µg/ml</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td>15.625µg/ml</td>
</tr>
<tr>
<td>Metronidazole</td>
<td><em>S. mutans</em></td>
<td>500µg/ml</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus</em></td>
<td>500µg/ml</td>
</tr>
<tr>
<td></td>
<td><em>E. faecalis</em></td>
<td>500µg/ml</td>
</tr>
<tr>
<td></td>
<td><em>P. gingivalis</em></td>
<td>0.975µg/ml</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td>1µg/ml</td>
</tr>
</tbody>
</table>

^5^ - Part of published article of this research protocol
TABLE 2b : MIC of combination of antimicrobial substances by broth dilution method $ ^{\$} $

<table>
<thead>
<tr>
<th>Test Agent</th>
<th>Microorganism</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin + Metronidazole</td>
<td>S. mutans</td>
<td>1.95µg/ml</td>
</tr>
<tr>
<td></td>
<td>S. aureus</td>
<td>7.81µg/ml</td>
</tr>
<tr>
<td></td>
<td>E. faecalis</td>
<td>1.95µg/ml</td>
</tr>
<tr>
<td></td>
<td>P. gingivalis</td>
<td>0.039µg/ml</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>1.95µg/ml</td>
</tr>
<tr>
<td>Amoxicillin clavulanate + Metronidazole</td>
<td>S. mutans</td>
<td>3.90µg/ml</td>
</tr>
<tr>
<td></td>
<td>S. aureus</td>
<td>15.625µg/ml</td>
</tr>
<tr>
<td></td>
<td>E. faecalis</td>
<td>15.625µg/ml</td>
</tr>
<tr>
<td></td>
<td>P. gingivalis</td>
<td>0.019µg/ml</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>15.625µg/ml</td>
</tr>
</tbody>
</table>

$ ^{\$} $ - Part of published article of this research protocol
### TABLE 2c: Antimicrobial activity of the endodontic medicaments and vehicle combinations against selected pathogens using agar well diffusion method

<table>
<thead>
<tr>
<th>Antimicrobial drugs</th>
<th>Vehicles</th>
<th>$S. \text{ mutans}$ (mm)</th>
<th>$S. \text{ aureus}$ (mm)</th>
<th>$E. \text{ faecalis}$ (mm)</th>
<th>$P. \text{ gingivalis}$ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorhexidine</td>
<td>PEG</td>
<td>31.00 (1.00)</td>
<td>39.00 (1.00)</td>
<td>31.67 (1.52)</td>
<td>26.00 (1.00)</td>
</tr>
<tr>
<td></td>
<td>PG</td>
<td>32.00 (1.00)</td>
<td>37.67 (1.15)</td>
<td>29.33 (0.57)</td>
<td>27.00 (1.00)</td>
</tr>
<tr>
<td></td>
<td>PG +PEG</td>
<td>31.33 (0.57)</td>
<td>37.33 (1.52)</td>
<td>29.67 (1.52)</td>
<td>27.33 (0.57)</td>
</tr>
<tr>
<td></td>
<td>Glycerine</td>
<td>31.33 (0.57)</td>
<td>35.67 (0.57)</td>
<td>28.00 (1.00)</td>
<td>27.67 (1.52)</td>
</tr>
<tr>
<td></td>
<td>H-Value</td>
<td>1.96</td>
<td>7.30</td>
<td>6.79</td>
<td>3.37</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.58 (NS)</td>
<td>0.063 (NS)</td>
<td>0.079 (NS)</td>
<td>0.337 (NS)</td>
</tr>
<tr>
<td>C+M</td>
<td>PEG</td>
<td>-</td>
<td>25.00 (1.00)</td>
<td>34.00 (1.00)</td>
<td>37.00 (1.00)</td>
</tr>
<tr>
<td></td>
<td>PG</td>
<td>-</td>
<td>24.00 (1.00)</td>
<td>33.00 (1.00)</td>
<td>38.33 (0.57)</td>
</tr>
<tr>
<td></td>
<td>PG +PEG</td>
<td>-</td>
<td>24.00 (1.00)</td>
<td>33.00 (1.00)</td>
<td>39.00 (1.00)</td>
</tr>
<tr>
<td></td>
<td>Glycerine</td>
<td>-</td>
<td>23.67 (0.57)</td>
<td>32.67 (0.57)</td>
<td>32.33 (1.52)</td>
</tr>
<tr>
<td></td>
<td>H-Value</td>
<td>3.041</td>
<td>3.041</td>
<td>9.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.385 (NS)</td>
<td>0.385 (NS)</td>
<td>0.029*</td>
<td></td>
</tr>
<tr>
<td>A+M</td>
<td>PEG</td>
<td>25.00 (1.00)</td>
<td>27.00 (1.00)</td>
<td>26.00 (1.00)</td>
<td>46.00 (1.00)</td>
</tr>
<tr>
<td></td>
<td>PG</td>
<td>26.33 (0.57)</td>
<td>27.00 (1.00)</td>
<td>25.33 (1.52)</td>
<td>46.33 (1.52)</td>
</tr>
<tr>
<td></td>
<td>PG +PEG</td>
<td>25.33 (2.08)</td>
<td>26.67 (1.52)</td>
<td>27.33 (.57)</td>
<td>46.00 (1.00)</td>
</tr>
<tr>
<td></td>
<td>Glycerine</td>
<td>20.00 (1.00)</td>
<td>25.00 (1.00)</td>
<td>28.00 (1.00)</td>
<td>45.00 (1.00)</td>
</tr>
<tr>
<td></td>
<td>H-Value</td>
<td>7.56</td>
<td>4.44</td>
<td>6.72</td>
<td>2.11</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.056 (NS)</td>
<td>0.217 (NS)</td>
<td>0.081 (NS)</td>
<td>0.548 (NS)</td>
</tr>
</tbody>
</table>

#Kruskal Wallis test

*p<0.05 significant  
*p>0.05 non significant, NS

a pairwise comparison using post hoc Mann Whitney U test statistically significant (p<0.05). All other pairwise comparisons are non significant (NS)

- Part of published article of this research protocol
Phase 1:

C. *In vitro* Antibacterial activity of innovative endodontic medicaments and
different vehicle combinations by agar well diffusion method using
commercial drugs

Table 3: *In vitro* Antibacterial activity of innovative endodontic medicaments
and different vehicle combinations against ATCC strains by agar well diffusion
method using commercial drugs

| Drug     | Vehicles |  | S. mutans | S. aureus | E. faecalis | E. coli | N |
|----------|----------| |          |           |            |          |   |
|          |          | Mean±SD (Zones of Inhibition in mms ) |          |            |          |          |   |
| CHX(2%)  | PEG      | 20.33±0.577 | 21.33±0.577 | 21.33±1.155 | 21.00±0.000 | 3 |
|          | PG       | 20.00±0.000 | 20.33±0.577 | 20.67±0.577 | 20.00±0.000 | 3 |
|          | PG+PEG   | 20.67±0.577 | 20.33±0.577 | 21.67±0.577 | 21.33±0.577 | 3 |
|          | Glycerine| 20.00±0.000 | 20.33±0.577 | 21.00±0.000 | 20.00±0.000 | 3 |
|          | Total    | 20.25±0.452 | 20.58±0.669 | 21.17±0.718 | 20.58±0.669 | 12 |
| C+M      | PEG      | 22.00±1.000 | 20.67±0.577 | 25.67±0.577 | 25.00±0.000 | 3 |
|          | PG       | 21.67±0.577 | 22.67±0.577 | 24.33±0.577 | 25.00±0.000 | 3 |
|          | PG+PEG   | 19.67±0.577 | 22.00±1.000 | 26.00±0.000 | 24.00±0.000 | 3 |
|          | Glycerine| 22.33±0.577 | 23.33±0.577 | 26.00±1.000 | 22.67±0.577 | 3 |
|          | Total    | 21.42±1.240 | 22.17±1.193 | 25.50±0.905 | 24.17±1.030 | 12 |
| A+M      | PEG      | 32.67±0.577 | 31.00±0.1000 | 28.33±0.577 | 30.67±1.155 | 3 |
|          | PG       | 33.00±0.000 | 31.33±0.577 | 31.33±0.577 | 32.33±1.528 | 3 |
|          | PG+PEG   | 31.33±0.577 | 31.00±0.000 | 30.67±0.577 | 30.67±0.577 | 3 |
|          | Glycerine| 31.00±1.000 | 33.67±0.577 | 30.67±0.577 | 30.00±1.000 | 3 |
|          | Total    | 32.00±1.044 | 31.75±1.288 | 30.25±1.288 | 30.92±1.311 | 12 |
Phase 2:
A. **Primary Objective: Ex-Vivo Evaluation of The Antimicrobial Activity**

Table 4: Organisms detected and their association with signs and symptoms present in subjects

<table>
<thead>
<tr>
<th>Org Detected</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Ps</td>
<td>0</td>
<td>3(50.0%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3(50.0%)</td>
</tr>
<tr>
<td>Tp</td>
<td>0</td>
<td>5(83.3%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1(16.7%)</td>
</tr>
<tr>
<td>S</td>
<td>0</td>
<td>2(33.3%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4(66.7%)</td>
</tr>
<tr>
<td>Ds</td>
<td>0</td>
<td>5(83.3%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1(16.7%)</td>
</tr>
<tr>
<td>V</td>
<td>0</td>
<td>6(100%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>NV</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6(100%)</td>
</tr>
<tr>
<td>X</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6(100%)</td>
</tr>
</tbody>
</table>

Fishers exact test, *p<0.05 statistically significant, p>0.05 non significant, NS

Where, Ps- Pain on stimulus,
Tp - Tender on percussion
S- Presence or History of swelling,
Ds - Draining sinus present
V- Vital pulp tissue in the root canal,
NV- Non-vital pulp tissue in the root canal
X- Periapical radiolucenty on radiograph.

1.0 All organisms present
2.0 *Streptococcus spp* present only
3.0 *Strep. Spp* and *Porphyromonas gingivalis* present
4. 0 *Strep. Spp* and *Enterococcus faecalis* present.
Results

Table 5a: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicle combinations against *Streptococcus spp*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean (SD)</th>
<th>Median (Q1-Q3)</th>
<th>Kruskal wallis test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chi-Square</td>
</tr>
<tr>
<td>C+M+G</td>
<td>28.75 (7.11)</td>
<td>30 (24-34)</td>
<td></td>
</tr>
<tr>
<td>A+M+G</td>
<td>31.32 (6.85)</td>
<td>32 (27-35)</td>
<td></td>
</tr>
<tr>
<td>CHX+G</td>
<td>23.21 (6.29)</td>
<td>22 (19-28)</td>
<td>193.638</td>
</tr>
<tr>
<td>C+M+PEG</td>
<td>26.03 (8.36)</td>
<td>28 (20-32)</td>
<td></td>
</tr>
<tr>
<td>A+M+PEG</td>
<td>31.95 (7.13)</td>
<td>32 (26-36)</td>
<td></td>
</tr>
<tr>
<td>CHX+PEG</td>
<td>23.84 (7.52)</td>
<td>24 (18-29)</td>
<td></td>
</tr>
<tr>
<td>C+M+PG</td>
<td>26.03 (7.82)</td>
<td>30 (24-32)</td>
<td></td>
</tr>
<tr>
<td>A+M+PG</td>
<td>32.26 (7.77)</td>
<td>32 (26-34)</td>
<td></td>
</tr>
<tr>
<td>CHX+PG</td>
<td>22.91 (7.71)</td>
<td>21 (18-30)</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05 statistically significant  p>0.05 non significant, NS

Table 5b: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicle combinations against *Porphyromonas gingivalis*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean (SD)</th>
<th>Median (Q1-Q3)</th>
<th>Kruskal wallis test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chi-Square</td>
</tr>
<tr>
<td>C+M+G</td>
<td>29.67 (8.41)</td>
<td>32 (22.25, 35.75)</td>
<td></td>
</tr>
<tr>
<td>A+M+G</td>
<td>35.75 (9.28)</td>
<td>34.5 (32.25, 42)</td>
<td></td>
</tr>
<tr>
<td>CHX+G</td>
<td>21.15 (6.21)</td>
<td>23 (17, 25)</td>
<td></td>
</tr>
<tr>
<td>C+M+PEG</td>
<td>29.75 (7.39)</td>
<td>31 (28, 34)</td>
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<td>35.25 (7.61)</td>
<td>37 (33, 39.75)</td>
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</tr>
<tr>
<td>CHX+PEG</td>
<td>22.74 (5.79)</td>
<td>23 (20, 25)</td>
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</tr>
<tr>
<td>C+M+PG</td>
<td>30.42 (7.01)</td>
<td>30 (27.25, 34)</td>
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<td>A+M+PG</td>
<td>39.37 (9.27)</td>
<td>42 (36.25, 44.75)</td>
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</tr>
<tr>
<td>CHX+PG</td>
<td>20.67 (5.41)</td>
<td>20 (18, 24)</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05 statistically significant  p>0.05 non significant, NS
Table 5c: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicles combinations against *Enterococcus faecalis*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean (SD)</th>
<th>Median (Q1-Q3)</th>
<th>Kruskal wallis test</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td>Chi-Square</td>
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<td>29 (24-37)</td>
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<td>A+M+G</td>
<td>33.58 (7.24)</td>
<td>33 (30-40)</td>
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</tr>
<tr>
<td>CHX+G</td>
<td>26.58 (6.31)</td>
<td>30 (21-32)</td>
<td></td>
</tr>
<tr>
<td>C+M+PEG</td>
<td>28.47 (6.96)</td>
<td>31 (22-34)</td>
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</tr>
<tr>
<td>A+M+PEG</td>
<td>34.53 (7.34)</td>
<td>34 (33-40)</td>
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</tr>
<tr>
<td>CHX+PEG</td>
<td>25.68 (7.17)</td>
<td>29 (19-32)</td>
<td></td>
</tr>
<tr>
<td>C+M+PG</td>
<td>31.11 (10.66)</td>
<td>29 (28-42)</td>
<td></td>
</tr>
<tr>
<td>A+M+PG</td>
<td>33.79 (8.54)</td>
<td>32 (29-42)</td>
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</tr>
<tr>
<td>CHX+PG</td>
<td>25.16 (8.46)</td>
<td>28 (17-30)</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05 statistically significant  p>0.05 non significant, NS
### Results

Table 6a: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each vehicle against *Streptococcus spp*

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Medicament</th>
<th>N</th>
<th>Mean (SD)</th>
<th>Median (Q1-Q3)</th>
<th>Kruskall Wallis test</th>
<th>Chi square value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>C+M</td>
<td>111</td>
<td>28.75 (7.11)</td>
<td>30 (24-34)</td>
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<td>67.87</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>A+M</td>
<td>111</td>
<td>31.32 (6.85)</td>
<td>32 (27-35)</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>CHX</td>
<td>111</td>
<td>23.21 (6.29)</td>
<td>22 (19-28)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PEG</td>
<td>C+M</td>
<td>111</td>
<td>26.03 (8.36)</td>
<td>28 (20-32)</td>
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<td>57.30</td>
<td>&lt;0.001*</td>
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<td>111</td>
<td>31.95 (7.13)</td>
<td>32 (26-36)</td>
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<tr>
<td></td>
<td>CHX</td>
<td>111</td>
<td>23.84 (7.52)</td>
<td>24 (18-29)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>C+M</td>
<td>111</td>
<td>28.60 (7.82)</td>
<td>30 (24-32)</td>
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<td>69.16</td>
<td>&lt;0.001*</td>
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<td>111</td>
<td>32.26 (7.77)</td>
<td>32 (26-34)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CHX</td>
<td>111</td>
<td>22.91 (7.71)</td>
<td>21 (18-30)</td>
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*p<0.05 statistically significant, p>0.05 non significant, NS

Table 6b: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each vehicle against *P. gingivalis*

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Medicament</th>
<th>N</th>
<th>Mean (SD)</th>
<th>Median (Q1-Q3)</th>
<th>Kruskall Wallis test</th>
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</thead>
<tbody>
<tr>
<td>G</td>
<td>C+M</td>
<td>24</td>
<td>29.67 (8.41)</td>
<td>32 (22.25, 35.75)</td>
<td>29.78 &lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>A+M</td>
<td>24</td>
<td>35.75 (9.28)</td>
<td>34.5 (32.25, 42)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CHX</td>
<td>27</td>
<td>21.15 (6.21)</td>
<td>23 (17, 25)</td>
<td></td>
</tr>
<tr>
<td>PEG</td>
<td>C+M</td>
<td>24</td>
<td>29.75 (7.39)</td>
<td>31 (28, 34)</td>
<td>31.43 &lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>A+M</td>
<td>24</td>
<td>35.25 (7.61)</td>
<td>37 (33, 39.75)</td>
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</tr>
<tr>
<td></td>
<td>CHX</td>
<td>27</td>
<td>22.74 (5.79)</td>
<td>23 (20, 25)</td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>C+M</td>
<td>24</td>
<td>30.42 (7.01)</td>
<td>30 (27.25, 34)</td>
<td>42.33 &lt;0.001*</td>
</tr>
<tr>
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<td>A+M</td>
<td>24</td>
<td>39.37 (9.27)</td>
<td>42 (36.25, 44.75)</td>
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<tr>
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<td>CHX</td>
<td>27</td>
<td>20.67 (5.41)</td>
<td>20 (18, 24)</td>
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</tr>
</tbody>
</table>

*p<0.05 statistically significant, p>0.05 non significant, NS
### Table 6c (Diagram 2c): Comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each vehicle against *E. faecalis*

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Medicament</th>
<th>N</th>
<th>Mean (SD)</th>
<th>Median (Q1-Q3)</th>
<th>Kruskal Wallis test</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
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<td></td>
<td></td>
<td>p-value</td>
</tr>
<tr>
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<td>C+M</td>
<td>19</td>
<td>30.21 (7.25)</td>
<td>29 (24-37)</td>
<td>8.014</td>
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<tr>
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<td>A+M</td>
<td>19</td>
<td>33.58 (7.24)</td>
<td>33 (30-40)</td>
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</tr>
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<td>CHX</td>
<td>19</td>
<td>26.58 (6.31)</td>
<td>30 (21-32)</td>
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<td>C+M</td>
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<td>31 (22-34)</td>
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<td>CHX</td>
<td>19</td>
<td>25.68 (7.17)</td>
<td>29 (19-32)</td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>C+M</td>
<td>19</td>
<td>31.11 (10.66)</td>
<td>29 (28-42)</td>
<td>9.751</td>
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<td>A+M</td>
<td>19</td>
<td>33.79 (8.54)</td>
<td>32 (29-42)</td>
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</tr>
<tr>
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<td>CHX</td>
<td>19</td>
<td>25.16 (8.46)</td>
<td>28 (17-30)</td>
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</tr>
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*p<0.05 statistically significant, p>0.05 non significant, NS

### Table 6d: Post hoc comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each of the three vehicles against selected pathogens

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Medicament 1(C+M) vs 2(A+M)</th>
<th>Medicament 1(C+M) vs 3(CHX)</th>
<th>Medicament 2(A+M) vs 3(CHX)</th>
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<tbody>
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<td>U statistic</td>
<td>p-value</td>
<td>U statistic</td>
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<td></td>
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<tr>
<td>Streptococcus spp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>4909.50</td>
<td>0.009*</td>
<td>3455.50</td>
</tr>
<tr>
<td>PEG</td>
<td>3684.50</td>
<td>&lt;0.001*</td>
<td>5098.50</td>
</tr>
<tr>
<td>PG</td>
<td>4544.50</td>
<td>0.001*</td>
<td>3633.00</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>177.50</td>
<td>0.02*</td>
<td>142.00</td>
</tr>
<tr>
<td>PEG</td>
<td>137.50</td>
<td>0.002*</td>
<td>131.00</td>
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<tr>
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<td>93.50</td>
<td>&lt;0.001*</td>
<td>71.50</td>
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<tr>
<td>E. faecalis</td>
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</tr>
<tr>
<td>G</td>
<td>131.50</td>
<td>0.15(NS)</td>
<td>131.50</td>
</tr>
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<tr>
<td>PG</td>
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<td>0.39(NS)</td>
<td>107.50</td>
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</tbody>
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Mann Whitney U test, *p<0.05 statistically significant, p>0.05 non significant, NS
Table 7a: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each medicament against *Streptococcus spp*

<table>
<thead>
<tr>
<th>Medicament</th>
<th>Vehicle</th>
<th>N</th>
<th>Mean (SD)</th>
<th>Median (Q1-Q3)</th>
<th>Kruskall Wallis test</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Chi square value</td>
</tr>
<tr>
<td>C+M</td>
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<td>111</td>
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<td>PEG</td>
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<td>26.03 (8.36)</td>
<td>28 (20-32)</td>
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</tr>
<tr>
<td></td>
<td>PG</td>
<td>111</td>
<td>28.60 (7.82)</td>
<td>30 (24-32)</td>
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</tr>
<tr>
<td>A+M</td>
<td>G</td>
<td>111</td>
<td>31.32 (6.85)</td>
<td>32 (27-35)</td>
<td>0.16</td>
</tr>
<tr>
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<td>PEG</td>
<td>111</td>
<td>31.95 (7.13)</td>
<td>32 (26-36)</td>
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</tr>
<tr>
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<td>PG</td>
<td>111</td>
<td>32.26 (7.77)</td>
<td>32 (26-34)</td>
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</tr>
<tr>
<td>CHX</td>
<td>G</td>
<td>111</td>
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<td>22 (19-28)</td>
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<td>23.84 (7.52)</td>
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</tr>
<tr>
<td></td>
<td>PG</td>
<td>111</td>
<td>22.91 (7.71)</td>
<td>21 (18-30)</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05 statistically significant,  
*p>0.05 non significant, NS

Table 7b: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each medicament against *P. gingivalis*

<table>
<thead>
<tr>
<th>Medicament</th>
<th>Vehicle</th>
<th>N</th>
<th>Mean (SD)</th>
<th>Median (Q1-Q3)</th>
<th>Kruskall Wallis test</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Chi square value</td>
</tr>
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<td>32 (22.25-35.75)</td>
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<td>PEG</td>
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<td>29.75 (7.39)</td>
<td>31 (28-34)</td>
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<tr>
<td></td>
<td>PG</td>
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<td>30.42 (7.01)</td>
<td>30 (27.25-34)</td>
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</tr>
<tr>
<td>A+M</td>
<td>G</td>
<td>24</td>
<td>35.75 (9.28)</td>
<td>34.5 (32.25-42)</td>
<td>6.73</td>
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<td>37 (33-39.75)</td>
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<td>39.37 (9.27)</td>
<td>42 (36.25-44.75)</td>
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<td>21.15 (6.21)</td>
<td>23 (17-25)</td>
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<td>22.74 (5.79)</td>
<td>23 (20-25)</td>
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</tr>
<tr>
<td></td>
<td>PG</td>
<td>27</td>
<td>20.67 (5.41)</td>
<td>20 (18-24)</td>
<td></td>
</tr>
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</table>

*p<0.05 statistically significant,  
*p>0.05 non significant, NS
Table 7c: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each medicament against *E. faecalis*

<table>
<thead>
<tr>
<th>Medicament</th>
<th>Vehicle</th>
<th>N</th>
<th>Mean (SD)</th>
<th>Median (Q1-Q3)</th>
<th>Kruskall Wallis test</th>
<th>Chi square value</th>
<th>p-value</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>C+M</td>
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<td>29 (24-37)</td>
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<td>0.66(NS)</td>
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<td>PEG</td>
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<td>28.47 (6.96)</td>
<td>31 (22-34)</td>
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</tr>
<tr>
<td></td>
<td>PG</td>
<td>19</td>
<td>31.11 (10.66)</td>
<td>29 (28-42)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A+M</td>
<td>G</td>
<td>19</td>
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<td>33 (30-40)</td>
<td>0.546</td>
<td>0.76(NS)</td>
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<td>34 (33-40)</td>
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<tr>
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<td>PG</td>
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<td>33.79 (8.54)</td>
<td>32 (29-42)</td>
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</tr>
<tr>
<td>CHX</td>
<td>G</td>
<td>19</td>
<td>26.58 (6.31)</td>
<td>30 (21-32)</td>
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<td>0.79(NS)</td>
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</tr>
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<tr>
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<td>PG</td>
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<td>25.16 (8.46)</td>
<td>28 (17-30)</td>
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</table>

*p<0.05 statistically significant,  p>0.05 non significant, NS

Table 7d: Post hoc comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each of the three medicaments against selected pathogens

<table>
<thead>
<tr>
<th>Medicament</th>
<th>Vehicle</th>
<th>U statistic</th>
<th>p-value</th>
<th>U statistic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Streptococcus spp (C+M)</td>
<td>Porphyromonas gingivalis (A+M)</td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
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<td>Vehicle G vs PEG</td>
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</tr>
<tr>
<td>Vehicle G vs PG</td>
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<td>0.87(NS)</td>
<td>198.50</td>
<td>0.06(NS)</td>
<td></td>
</tr>
<tr>
<td>Vehicle PEG vs PG</td>
<td>5159.50</td>
<td>0.04*</td>
<td>162.00</td>
<td>0.009*</td>
<td></td>
</tr>
</tbody>
</table>

Mann Whitney U test,  *p<0.05 statistically significant,  p>0.05 non significant, NS
Phase 2:

B. Secondary Objective- PCR assay

Table 8a : Comparison of detection of *E. faecalis* by PCR and culture

<table>
<thead>
<tr>
<th>Ef by culture * Ef by PCR Crosstabulation</th>
<th>Ef by PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Count</td>
<td>59</td>
<td>34</td>
</tr>
<tr>
<td>% of Total</td>
<td>52.7%</td>
<td>30.4%</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>% of Total</td>
<td>9.8%</td>
<td>7.1%</td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td>42</td>
</tr>
<tr>
<td>% of Total</td>
<td>62.5%</td>
<td>37.5%</td>
</tr>
</tbody>
</table>

McNemar test: p<0.001*

Table 8b : Comparison of detection of *P. gingivalis* by PCR and culture

<table>
<thead>
<tr>
<th>Pg by culture * Pg by PCR Crosstabulation</th>
<th>Pg by PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Count</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>% of Total</td>
<td>23.2%</td>
<td>25.0%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>55</td>
</tr>
<tr>
<td>% of Total</td>
<td>2.7%</td>
<td>49.1%</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>83</td>
</tr>
<tr>
<td>% of Total</td>
<td>25.9%</td>
<td>74.1%</td>
</tr>
</tbody>
</table>

McNemar test: p<0.001*
Phase 2:

Pie Chart 1: Tooth wise distribution of selected deciduous necrotic teeth for endodontic sample collection

Diagram 1a: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicle combinations against Streptococcus spp

<table>
<thead>
<tr>
<th>Study Groups</th>
<th>Zone of Inhibition in mms</th>
</tr>
</thead>
<tbody>
<tr>
<td>C+M+G</td>
<td>28.75</td>
</tr>
<tr>
<td>A+M+G</td>
<td>31.32</td>
</tr>
<tr>
<td>CHX+G</td>
<td>23.21</td>
</tr>
<tr>
<td>C+M+PEG</td>
<td>26.03</td>
</tr>
<tr>
<td>A+M+PEG</td>
<td>31.95</td>
</tr>
<tr>
<td>CHX+PEG</td>
<td>23.84</td>
</tr>
<tr>
<td>C+M+PG</td>
<td>28.6</td>
</tr>
<tr>
<td>A+M+PG</td>
<td>32.26</td>
</tr>
<tr>
<td>CHX+PG</td>
<td>22.91</td>
</tr>
</tbody>
</table>

*p<0.001
Diagram 1b: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicles combinations against *Porphyromonas gingivalis*

![Diagram showing comparison of antimicrobial activity against Porphyromonas gingivalis](image)

* p<0.001

Diagram 1c: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicles combinations against *Enterococcus faecalis*

![Diagram showing comparison of antimicrobial activity against Enterococcus faecalis](image)

* p<0.001
Results

Diagram 2a: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each vehicle against *Streptococcus* spp

Diagram 2b: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each vehicle against *P. gingivalis*
Diagram 2c: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each vehicle against *E. faecalis*

Diagram 3a: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each medicament against *Streptococcus spp*
Diagram 3b: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each medicament against *P. gingivalis*

*\( p=0.034 \)

Diagram 3c: Comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each medicament against *E. faecalis*

* \( p>0.05 \) (NS)
DISCUSSION

Phase 1 *in vitro* studies were carried out against ATCC strains of selected bacteria (*Streptococcus mutans, Staphylococcus aureus, Enterococcus faecalis, and Escherchia coli*) which are commensals of infected root canals of teeth. Later *in vitro* study was carried out using pure drugs and commercial preparations by agar well diffusion. Following is the discussion of the results of Phase 1 *in vitro* part of the study.

Phase 1:

A. *In vitro* Determination of Bactericidal activity of vehicles against ATCC strains by broth dilution

Successful preservation of tooth depends on response to the conventional therapy in the pediatric and adult endodontics. These refractory cases fail due to many reasons like anatomic variation, microbial biofilms and antibiotic resistance of resident microorganisms. To overcome the global menace of antibiotic resistance and development of new antibiotics, the use of alternative medications and substances for instance herbal, or the combination of pharmaceutical excipients is suggested to increase the spectrum of antimicrobial action. The most virulent microorganisms are the facultative anaerobes as they develop resistance.

Intracanal medicaments are necessary in endodontics as necrotic and abscessed teeth are devoid of blood circulation. Systemic antibiotics fail to reach the site of infection via the blood supply and are ineffective. Also, local drug delivery and sustained release of endodontic medicaments are advantageous due to better diffusion into the periradicular tissues. The release, onset of action, dissociation and diffusion of the endodontic medicaments through dentinal tubules is directly
related to the vehicles or carriers utilized. Glycols like monopropylene, dipropylene and triethylene has been investigated extensively for their bactericidal activity. Vaamonde et al (1982) and Chirfe et al (1983) investigated the bactericidal activity of PEG 400 and Carreira et al (2007) investigated antimicrobial effect of PEG 1000. Antimicrobial activity of PEG 1000 may be due hydrophilic property of PEG which inhibits microbial growth due to loss of water, as water is required for microbial multiplication and development. PEG 1000 in combination with endodontic medicaments like propylene glycol, also enhanced dentinal penetration. Due to lack of a study comparing the bactericidal activity of these excipients; the first of the three in vitro studies (Phase 1 A.) was executed.

Propylene glycol is a known antimicrobial and is an effective preservative. Carreira et al (2007) focused synergism in bactericidal activity which was observed in the Ciprofloxacin-PEG 1000 association, Metronidazole-PEG 1000 association and Ciprofloxacin-Metronidazole-PEG 1000 combination. Also, PEG 400 produced severe plasmolysis, cell-wall collapse and finger-like extrusions in Klebsiella pneumoniae. Promising results have been shown by PEG-coated nanoparticles being most effective in killing Eschericia coli, Staphylococcus aureus and multi-drug resistant clinical isolates of Shigella spp and Vibrio cholera. Of all the vehicles being used in endodontics camphorated monochloro phenol(CMCP) is effective but is tissue toxic whereas distilled water possess no antimicrobial activity.

In our study, 100% concentration of all vehicles showed antimicrobial activity (Table 1). Micro-broth dilution was performed as diffusion through agar of these viscous vehicles is difficult. Enterococcus faecalis was most susceptible to propylene glycol but resistant to other vehicles. Olitzky in 1965 has reported that propylene glycol is a known antimicrobial with marked germicidal activity. Glycerine had
least antimicrobial activity at 100% concentration only, similar to observations of no activity by Gomes et al in 2002. Few authors noticed bactericidal activity at 30% and 25% when incubated for a longer time of 48 hrs to 7 days; in contrast to the findings of the present study.

100% concentration of all PEG 400, glycerine and PG combination with PEG 400 respectively showed similar bactericidal activity against all the selected organisms. 25% of PEG 1000 was bactericidal against *Streptococcus mutans* and *Eschericia coli*. 25% of PEG 1000 being bactericidal to *Streptococcus mutans* and *Eschericia coli* is similar with the findings by Carreira et al. *Staphylococcus aureus* and *Enterococcus faecalis* vary in their susceptibility due to different ATCC strains selected for this in vitro study. Antimicrobial activity of PEG 400 and glycerine are in contrast to the findings of Gomes et al as they have used agar diffusion method. The viscosity of the vehicles hinders the diffusion through agar and absence of zones of inhibition does not depict lack of antimicrobial activity. Both PEG and propylene glycol have low toxic potential and are common carriers for drugs. Also, they enhance handling properties of drugs and aid in ease of placement. Combination of PG and PEG 400 exhibited no synergistic effect. PEG 400 is advantageous in not interacting with other components, antibacterial activity and a low water activity.
**Table 9**: Following are the list of authors with findings in accordance and contrary to our research related to antimicrobial activity and toxicity of vehicles namely, Glycerine, Polyethylene glycol 400 (PEG 400):

<table>
<thead>
<tr>
<th>Vehicles studied / Authors</th>
<th>Method / Organism Tested</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Glycerine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Barr M and Tice L, 1971¹⁴⁶</td>
<td>Broth Dilution Test / Multiple Tube Dilution Test ATCC strains of <em>Pseudomonas aeruginosa</em>, <em>Escherihia coli</em>, <em>Salmonella typhi</em>, <em>Staphylococcus aureus</em>.</td>
<td>Possesses antimicrobial activity. Bacteriostatic for <em>Salmonella typhi</em> at 37.8% after 48 hrs and bactericidal at 23.6% after 7 days</td>
</tr>
<tr>
<td>2) Gomes et al, 2002³²</td>
<td>Modified agar diffusion test</td>
<td>No antimicrobial effect.</td>
</tr>
<tr>
<td><strong>II. PEG 400</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Vaamonde et al, 1982¹⁵¹</td>
<td>Agar Dilution Method <em>Staphylococcus aureus</em>.</td>
<td>Significant inhibitory effect independent of water activity ($a_w$) so, good antimicrobial activity.</td>
</tr>
<tr>
<td>2) Chirfe et al, 1983¹⁴¹</td>
<td>Agar Dilution Method <em>S. aureus</em>, <em>Klebshiella pneumoniae</em>, <em>P. aeroginosa</em>, <em>E. coli</em></td>
<td>Significant antibacterial activity due to lowering $a_w$ and phase–contrast microscopy revealed clumping and morphological changes.</td>
</tr>
<tr>
<td>3) Bozzini JP et al, 1986¹⁴³</td>
<td>Electron Microscopy <em>Klebshiella pneumoniae</em></td>
<td>Severe plasmolysis in <em>K. pneumoniae</em> cells, cell wall collapse and fingerlike extrusions to</td>
</tr>
</tbody>
</table>
### Vehicles studied / Authors

<table>
<thead>
<tr>
<th>Method / Organism Tested</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicles studied</strong></td>
<td><strong>Method / Organism Tested</strong></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>4) Ambrose et al. 1991⁵²</td>
<td>Agar Dilution Method Clinical isolates of <em>S. aureus</em>, <em>S. epidermis</em>, <em>E. faecalis</em>, <em>E. coli</em>, group C β-hemolytic <em>Streptococcus</em>, <em>P. mirabilis</em> and <em>Klebsiella</em> spp.</td>
</tr>
<tr>
<td></td>
<td>Modified agar diffusion test</td>
</tr>
<tr>
<td>6) Li BQ et al, 2011¹⁴⁹</td>
<td>Systemic toxicity and toxicokinetics of a high dose of polyethylene glycol 400 in dogs following iv injection.</td>
</tr>
</tbody>
</table>

### III. PEG 1000

<table>
<thead>
<tr>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Of all the vehicles, PEG 1000 maximum antimicrobial activity, allows greater penetration. Antimicrobial action may be related to the hydrophilic property of PEG, removing water necessary for microbial growth.</td>
</tr>
<tr>
<td>Vehicles studied / Authors</td>
</tr>
<tr>
<td>----------------------------</td>
</tr>
<tr>
<td>IV. PG</td>
</tr>
<tr>
<td>1) Olitzky et al, 1965(^{145})</td>
</tr>
<tr>
<td>2) Kinnunen et al, 1991(^{142})</td>
</tr>
<tr>
<td>3) Rowe et al, 2009(^{147}) PG and PEG</td>
</tr>
</tbody>
</table>

### B. In vitro Antibacterial activity of endodontic medicaments and vehicle combinations against ATCC strains by agar well diffusion method using pure drugs

Hence, following bactericidal activity of vehicles, the promising vehicles were considered for the second *in vitro* study (Phase 1 B.) in combination with the intracanal medicaments in pure form. Initially MIC was carried out for single drug (Table 2a) followed by MIC of combination of drugs by broth dilution method respectively (Table 2b), lastly antibacterial activity of combination of pure drugs with
vehicles was assessed using agar well diffusion method (Table 2c). A dose of 100µg/ml was bactericidal, no bacteria were recovered from the samples in the previous studies. However, concentrations of 1µg/ml and 10µg/ml were bacteriostatic and allowed some microbes to persist.\(^{43,153}\)

Chlorhexidine has been used as irrigant and intracanal medicament in Endodontics.\(^{47,154}\) It has substantive properties, is a cationic biguanide and an antiseptic, and is biocompatible and less toxic at high concentrations.\(^{16,29}\) It is bacteriostatic at low concentration and bactericidal at high concentration exhibiting antimicrobial activity from 0.1% to 2%.\(^{16,29,155}\)

**Figure 2**: Mechanism of Action of CHX-Chlorhexidine being a cationic molecule adheres to the negatively charged cell wall of microorganisms thus, disrupts the cell-wall, osmotic balance, inducing changes and precipitation of cytoplasm leading to cell death.


In this study, Chlorhexidine had an MIC of 0.019% of against *P. gingivalis* and 0.078% against other facultative anaerobes with the highest concentration of
Discussion

0.156% against *E. faecalis*. Mistry et al observed similar results of <0.0625% of Chlorhexidine against facultative anaerobes but the same standard strain of *E. faecalis* was susceptible to higher values inspite of broth dilution being used to determine MIC in both studies.\(^{155}\) *P. gingivalis*, a strict anaerobe was most susceptible to Chlorhexidine using agar well technique; which is in accordance with the study by Filho et al.\(^ {118}\) Chlorhexidine showed lesser zones of inhibition of *P. gingivalis* when studied with vehicles by agar well diffusion method as compared to other microbes. This might be due to Chlorhexidine being lesser effective against gram negative organisms as compared to gram positive.\(^ {28}\)

MIC of Ciprofloxacin and reduction in MIC when combined with Metronidazole are similar with the results stated by Carreira et al except for *S. aureus*. The difference in results might be attributed to use of different ATCC strains.\(^ {106}\) The 5 µg/ml of Ciprofloxacin produced greater zones of inhibition against clinical isolates of *S. aureus*\(^ {156}\) and *E. faecalis*\(^ {157}\) as compared to 100 µg/ml of Ciprofloxacin in our study against standard strains of the same microorganisms. Jain et al had a similar observation of standard strain of *S. mutans* being resistant to C+M alongwith the four vehicles when tested by agar well diffusion method.\(^ {158}\) DAP had similar antimicrobial activity as TAP\(^ {73}\) and it was observed that MIC against all organisms except *E. faecalis* lowered when combination of C+M was used as compared to Ciprofloxacin alone. Choudhary et al, observed that Amoxycillin and Ciprofloxacin had greater antimicrobial activity as compared to tetracyclines against clinical isolates of *S. mutans*.\(^ {159}\)
Table 10: Following are the studies which have studied the antimicrobial activity and tissue toxicity of Chlorhexidine, Ciprofloxacin and Amoxycillin clavulanate and have findings supporting and contrary to our research observations:

<table>
<thead>
<tr>
<th>Vehicles studied / Authors</th>
<th>Method / Organism tested</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. 2% Chlorhexidine</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| 1) Mistry et al, 2014\(^{155}\) | Microbroth dilution method  
  *S. mutans*, *S. aureus* and *E. faecalis*  | Chlorhexidine is an effective antimicrobial with MIC<0.0625 % |
| 2) Gomes et al, 2006\(^{27}\) | Agar diffusion method and Direct contact test  
  ATCC strains of *S. aureus*, *C. albicans*, *E. faecalis*, *P. gingivalis*, *P. endodontalis*, *Prevotella intermedia*  | 2 % CHX gel alone demonstrated strongest antimicrobial action. |
| 3) Filho et al, 2008\(^{118}\) | Agar diffusion method and Direct contact test  
  ATCC strains of *S. mutans*, *S. sobrinus*, *C. albicans*, *E. faecalis* and clinical isolates of *P. gingivalis*, *Prevotella intermedia*.  | *S. mutans* showed significantly larger zones of inhibition as compared to *P. gingivalis*, *Prevotella intermedia*.  
  *C. albicans*, and *E. faecalis* were most resistant. |
## Discussion

<table>
<thead>
<tr>
<th>Vehilces studied / Authors</th>
<th>Method / Organism tested</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>II. Ciprofloxacin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Oboh IE et al, 2007(^{156})</td>
<td>Agar diffusion method Standard strain of \textit{S. aureus}, and clinical isolates of \textit{S. aureus}, \textit{C. albicans}, \textit{E. faecalis}, \textit{Klebsiella pneumoniae}, \textit{Pseudomonas aeruginosa}, \textit{Bacillus subtilis}, \textit{E. coli}</td>
<td>5 (\mu)g/ml showed higher antimicrobial activity as compared to the 100 (\mu)g/ml of Ciprofloxacin used in our study.</td>
</tr>
<tr>
<td>2) Jain et al, 2009(^{158})</td>
<td>Kirby-Bauer Disc diffusion method. Three Standard Strains of \textit{S. mutans}</td>
<td>\textit{S. mutans} showed resistance to Ciprofloxacin as in our study.</td>
</tr>
<tr>
<td><strong>III. Amoxycillin Clavulanate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Baumgartner et al, 2003(^{160})</td>
<td>E-test. Antibiotic susceptibility of 98 strains of bacteria isolated from 12 endodontic abscesses</td>
<td>Amoxycillin clavulanate was 100% effective against all 98 species of microorganisms.</td>
</tr>
</tbody>
</table>
Metronidazole had greatest resistance but if combined with Amoxicillin, sensitivity increased from 91% to 99%.

<table>
<thead>
<tr>
<th>Vehicles studied / Authors</th>
<th>Method / Organism tested</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>2) Salinas et al, 2006&lt;sup&gt;161&lt;/sup&gt;</td>
<td>-------------------------</td>
<td>Amoxicillin clavulanate had greatest sensitivity and lowest resistance amongst the commonly prescribed antibiotics followed by amoxicillin alone.</td>
</tr>
<tr>
<td>3) Ruparel et al, 2012&lt;sup&gt;69&lt;/sup&gt;</td>
<td>MTT assay SCAPs from 2 extracted immature mandibular third molars.</td>
<td>High concentrations of TAP, DAP and Augmentin (Amoxyccillin clavulanate) have a detrimental effect on SCAP survival.</td>
</tr>
</tbody>
</table>

Principles of Antibiotic Therapy advise the use of narrow spectrum and bactericidal drugs to prevent antibiotic resistance development.<sup>109</sup> Bactericidal drug combination overcome selective pressure and development of antibiotic resistance in microorganisms.<sup>85</sup> The most common reason for resistance in Enterobacteriaceae is β-lactamase production, so a β-lactamase inhibitor, clavulanic acid can be incorporated. So, Amoxycillin clavulanate, found to be 100% effective against endodontic bacteria<sup>160,161</sup> in comparison to Ciprofloxacin. In this in vitro study, modified DAP with Amoxycillin clavulanate was studied for the first time as an intracanal medicament.
Amoxycillin clavulanate has MIC values similar to the Indian Council Medical Research guidelines for *S. mutans* and *P. gingivalis*. Also, combination of Amoxycillin clavulanate with Metronidazole had MIC values, lower or similar for *S. mutans*, *E. faecalis*, *P. gingivalis* and *E. coli* except *S. aureus*. Antimicrobial activity of A+M when combined with the four different vehicles were the largest in terms of zones of inhibition against *P. gingivalis*.

*P. gingivalis*, an obligate anaerobe exhibits larger zones of inhibition as they are extremely susceptible to Metronidazole which targets anaerobes. 2% CHX exhibited antimicrobial activity against all selected pathogens contrary to the antibiotic medicaments used with an added advantage no resistance development.

PG when used as vehicle to carry intracanal medicaments improves the medicament penetration through dentinal tubules as first noted by Cruz et al. Carreira et al observed that PEG when used as vehicle to carry Metronidazole made resistant microbes sensitive. Vehicles improve handling properties of the resulting intracanal medicament paste and enhance the release of the intracanal medicaments from the paste. The results of the previous *in vitro* study (Phase 1 A.) highlight that all the selected vehicles possessed bactericidal activity.

Intracanal medicaments in combination with vehicles no significant difference in their antimicrobial activity except for PG which was significantly better than glycerine when used alongwith C+M intracanal medicament combination against *P. gingivalis* (Table 2b). PEG, PG+PEG and glycerine as vehicles with the three intracanal medicaments i.e., 2% Chlorhexidine gluconate, C+M and A+M might be lacking synergistic activity.

The agar well diffusion method used in this study is the most widely used method of antibacterial activity assessment of newer substances like plant extracts,
new drug formulations, dental materials, medicaments etc.\textsuperscript{164,165,166,155,27} This is one of the standard methods of comparing the antibacterial activity and the latest being the E-test. Limitation of E-test is that standardized strips for the combination of antimicrobial drugs with vehicles are unavailable, hence agar well diffusion method was performed in this \textit{in vitro} study.

C. \textbf{\textit{In vitro} Antibacterial activity of innovative endodontic medicaments and different vehicle combinations against ATCC strains by agar well diffusion method using commercial drugs}

It was observed from the previous \textit{in vitro} study (Phase 1 B.) that \textit{P. gingivalis}, an obligate anaerobe exhibits larger zones of inhibition as they are extremely susceptible to Metronidazole.\textsuperscript{163,167} Also, facultative anaerobes are the organisms which develop antibiotic resistance, hence \textit{Streptococcus mutans}, \textit{Staphylococcus aureus}, \textit{Enterococcus faecalis} and control as \textit{Escherichia coli} as per CLSI guidelines were selected. Combination of drugs with Metronidazole is done as per principles of antibiotic therapy\textsuperscript{109} and also, because endodontic infection in necrotic teeth is polymicrobial and predominantly anaerobic in nature.\textsuperscript{99,100,101} As for, commercial drugs as intracanal medicaments have been used \textit{in vivo} studies\textsuperscript{56,60,72}, hence this \textit{in vitro} study was carried out using commercial preparations. The commonly available tablets were used for the antibiotics i.e., 0.5 mg was tested as mentioned in the methodology section. 2\% CHX irrigant solution (Dentachlor), was used as the only available commercial preparation were irrigant solutions in India.

It was observed that all commercial preparations except A+M had lesser antimicrobial activity in terms of zones of inhibition as compared to the pure drugs.
Discussion

used in (Phase 1 B.). 2% CHX and C+M had lesser zones of inhibition respectively as compared to the ones in Table 2c. This might have been due to the fact that same drug concentrations utilized in the *in vitro* study in pure form has been not equal to drug concentration in 0.5mg of commercial drugs due to the presence of filler, binder and other substance used in the preparation of tablets from pure drugs. C+M was also effective against *S. mutans* contrary to the findings in Table 2c. Amongst all the three intracanal medicaments, A+M was the most effective irrespective of the vehicle utilized.  Amongst the vehicles it was interesting to note that either PG or PEG was the most effective vehicle with all the intracanal medicaments. Their combination i.e., PG+PEG did not have any synergistic effect hence, is not recommended as per this study. Glycerine had the lowest antibacterial activity hence, is not recommended as per the observations from this study. Hence, as per the findings of this study we suggest the combination of A+M with preferably with PG as vehicle and 2% CHX with PEG as vehicle. The combination of C+M had no predilection for the vehicle to be used to carry. The zones of inhibition were similar for C+M and 2% CHX with no difference due to use of the four different vehicles to carry them. Amongst all the medicaments A+M was most effective and with vehicle PG.

There are no studies comparing antibacterial activity of DAP consisting of combinations like A+M and C+M using commercially available preparations as in this study. Antibacterial activity of TAP has been assessed using agar well diffusion method and the zones of inhibition recorded are lower but comparable to the zones of inhibition of DAP(C+M) and modified DAP(A+M) recorded in our study. Agar well diffusion method was used inorder to compare the results to the study using *ex-vivo* model (Phase 2A.), to compare if diffusion of endodontic medicaments is hampered through dentinal tubules when used *in vivo*. 
Phase 2:

In this study though the distribution of males was higher (57%) in comparison to the female children (43%) but it has no influence on the organisms detected as organisms present depends on the environment present within the root canals. The deciduous mandibular molar teeth showed a higher distribution of necrotic pulp 28 (75%) as compared to deciduous maxillary molars 9 (25%) which is in accordance with the study by Fabris et al. This might be due to the fact that mandibular molars erupt earlier in the cavity than maxillary molars and are in function for longer period of time and due to food retention and hence, get carious more often.

A. Primary Objective: Antimicrobial activity of various combinations of intracanal medicaments and vehicles

Organisms detected and their association with signs and symptoms present in subjects

Table 4 depicts the organisms detected and their association with signs and symptoms present in subjects. As the organism detected were for an endodontic sample, the coding was done for all selected organisms detected in that particular sample. It means that if all the selected organisms were present i.e. Streptococcus spp, P. gingivalis and E. faecalis the code given was 1, followed by code 2 for detection of Streptococcus spp only, code 3 for detection of Streptococcus spp with P. gingivalis and code 4 for detection of Streptococcus spp with E. faecalis.

As organisms were always detected along with Streptococcus spp, the association with signs and symptoms is not true association with each organism but with the interaction between the organisms detected in each sample. Also, Treponema denticola was detected by means of PCR but was not considered as it was not
detected by culture. Previous studies have shown an association between \textit{T. denticola} and \textit{E. faecalis} with periapical radiolucency while \textit{P. gingivalis} with tenderness to percussion.\textsuperscript{103} When a total of 224 cultivable isolates were recovered belonging to 56 different bacterial species, association were observed between anaerobes especially gram negative microbes and pain, tenderness on percussion and swelling. \textit{P. gingivalis} was associated with pain, wet canals and tenderness to percussion; \textit{P. gingivalis} and \textit{E. faecalis} were associated with swelling and \textit{E. faecalis} and \textit{Streptococcus spp} was associated with previous endodontic treatment.\textsuperscript{97}

In our study no significant relation was observed between any of the organisms detected and the signs and symptoms recorded; similar to the findings by Siqueira JF et al that no particular signs and symptoms were positively associated with the presence of \textit{T. denticola} in root canals of carious, necrotic teeth with radiographic periradicular radiolucency. This might have been due to the reason for our association statistics to be not significant as we have only chosen teeth which have been diagnosed necrotic on the basis of periapical or furcation area radiolucency on radiograph. Also, in our study only the above mentioned selected organisms (\textit{Streptococcus spp}, \textit{P. gingivalis} and \textit{E. faecalis}) were detected and not all possible cultivable organisms hence the non-significant association. Though the association was statistically not significant, it is interesting to note that few organism combinations had higher percentage in relation to specific signs and symptoms.

For instance, history of pain was detected in 92.3\% of root canals detecting \textit{Streptococcus spp} and \textit{E. faecalis}, tenderness to percussion was detected in 32.7\% of root canals detecting \textit{Streptococcus spp} and \textit{P. gingivalis}\textsuperscript{97} which is in accordance with the study by Gomes et al. Swelling was detected in 66.7\% of root canals detecting all the selected microorganisms, followed by 55.8\% in root canals detecting.
Streptococcus spp and P. gingivalis\textsuperscript{97} which is in accordance with the study by Gomes et al but contrary to the findings by the same author in low association i.e., 23.1\% with root canals detecting Streptococcus spp and E. faecalis. Teeth with draining sinus had the highest percentage of 16.7\% in root canals detecting all the selected microorganisms which might be related to the fact that now the environment in the root canals becomes less anaerobic.

The findings of this study that vital root canals have lesser microbial load (27.7\%) as compared to non-vital root canals (72.3\%) further supported the findings by various authors.\textsuperscript{103,169,170} Lastly, as only teeth diagnosed as necrotic due to the presence of periradicular radiolucency were included in the study; association of organisms detected with it could not be estimated.

**Comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicle combinations against Streptococcus spp, P. gingivalis, and E. faecalis**

A+M+PG had the greatest zone of inhibiton i.e., 32.26 ±7.77 mms depicting maximum antimicrobial activity against Streptococcus spp, A+M+PG had greatest zone of inhibiton of 39.37 ± 9.27 mms against P. gingivalis and A+M+PEG had greatest zone of inhibiton of 34.53 ± 7.34 mms against E. faecalis. Amoxycillin Clavulanate being the most effective intracanal medicament is in accordance with the findings of Baumgartner et al, whereas PG and PEG being the ideal vehicles was pointed out by Cruz et al and Carreira et al respectively.\textsuperscript{81, 91,109,160}

To know exactly which intracanal medicament and vehicle were effective, comparisons were done between all three medicaments keeping vehicles constant and all three vehicles keeping medicaments constant. This would give the idea of the
effectiveness of each medicament and vehicle individually and which of the two to combine for maximum bactericidal effect and thus, achieve effective disinfection of the root canals of deciduous necrotic molars.

**Comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each vehicle against Streptococcus spp, P. gingivalis, and E. faecalis**

When comparison was done amongst the intracanal medicaments with the same vehicle, i.e., C+M, A+M, CHX in the same vehicle namely, glycerine followed by PEG and lastly, PG; it was observed that A+M had significantly more antimicrobial activity as compared to both C+M and CHX. These results are in accordance with study on antimicrobial susceptibility of endodontic organisms, which observed that Amoxicillin clavulanate was effective against most of the microorganisms especially anaerobes developing resistance.\(^\text{107,160}\) This has also been observed clinically in a case report using Amoxicillin in triple antibiotic paste instead of Minocycline to overcome the problem of discoloration inherent with tetracycline usage.\(^\text{80}\) Also, combination with Metronidazole could be more effective compared with administration of single drug.\(^\text{160,85,109}\) Also, study by Jardim et al showed that amoxicillin clavulanate was effective against β-lactams resistant isolates too except *Proteus spp*.

Similarly, when the same three intracanal medicaments were used with either PEG or PG, A+M was the most effective medicament irrespective of the vehicle used to dispense. Though there are no studies on using A+M as an intracanal medicament, it can be concluded from antibiotic susceptibility testing against endodontic pathogens that most effective is Amoxicillin clavulanate with no resistance\(^\text{160}\) Chunduri et al concluded that Amoxicillin still possesses activity against major pathogens in
Discussion

orofacial odontogenic infections but for severe infections Amoxicillin clavulanate is useful. The vehicles did not influence the antimicrobial activity against these organisms like *Streptococcus spp.*, and *P. gingivalis*. These observations were common to the selected organisms i.e. *Streptococcus spp.*, and *P. gingivalis*; as these organisms are also more susceptible as compared to *E. faecalis*. Also, *P. gingivalis* is more susceptible being an obligate anaerobe which is observed by the overall larger zones of inhibition and literature supports this observation.

When the same three intracanal medicaments were used with either glycerine, PEG or PG against *E. faecalis* A+M and C+M were similar in antimicrobial activity, the differences being statistically significant only when used with PEG. This shows that A+M can replace C+M i.e., the DAP and vehicles do play a role in enhancing the antibacterial efficacy especially against resistant organisms like *E. faecalis*. Also, these findings are in accordance to studies by Samuelsson DG et al (2000), ICMR Bulletin (2009), Al-Badah AS et al (2015) that *E. faecalis* is resistant to Cephalosporin, semi-synthetic penicillin, Ampicillin, Metronidazole and Tetracyclines etc thus supporting the combination of drugs used as in this study and antimicrobials like 2% Chlorhexidine. Numerous studies regarding antibiotic susceptibility of bacteria associated with endodontic, dentoalveolar and odontogenic infections have clinical evidence of amoxicillin clavulanate being most effective antibiotic.
Post hoc comparison of antimicrobial activity in terms of zones of inhibition (mms) of medicaments in each of the three vehicles against selected pathogens

C+M and CHX were similar in their antimicrobial activity; the differences being statistically significant only when used with PG. These observations suggest the use of CHX as an alternative antimicrobial agent instead of the C+M and A+M antibiotic combinations, though the difference between A+M and CHX was statistically significant. This is especially an important characteristic as *E. faecalis* is a resistant microbe. Use of antimicrobials like Chlorhexidine (CHX) also has added advantage of increasing shear bond strength when used as an intracanal medicament in young necrosed permanent teeth, not affecting the properties of the developing tooth root adversely. CHX also has low toxicity even at higher concentrations and its antibacterial activity increases as the concentration is increased.

Comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each medicament against *Streptococcus spp*, *P. gingivalis*, and *E. faecalis*

Glycerine and Propylene glycol (PG) were significantly better vehicles than PEG to carry (C+M) medicament against *Streptococcus spp*. Propylene glycol has been a ideal vehicle and has consistently showed good antimicrobial properties and also, when combined with intracanal medicaments throughout the various phases of the study (*in vitro* and *ex-vivo*). Though in the initial *in vitro* studies glycerine did not show promising results as a vehicle alone but when combined with intracanal medicaments especially antibiotic based medicaments, it possessed antimicrobial activity.
PG was significantly better vehicle as a carrier for (A+M) medicament than PEG and Glycerine when used against *P. gingivalis*. Hence PG can be recommended as the ideal vehicle to carry these medicaments against both *Streptococcus spp* and *P. gingivalis*. This is in accordance with the findings of Cruz et al (2002) and Ganesh MR et al (2014) though these studies did not compare it with other vehicles included in this study.

**Post hoc comparison of antimicrobial activity in terms of zones of inhibition (mms) of vehicles in each of the three medicaments against selected pathogens**

There is no significant difference between the use of vehicles in combination with the medicaments against *E. faecalis*. This is contrary to the findings of the previous part of our study where only vehicles were checked for their bactericidal activity against ATCC strains, and PG exhibited bactericidal activity at 25% against *E. faecalis* ATCC 35550. This points out to the fact that clinical strains might have developed resistance to bactericidal activity of vehicles. This is in accordance with many studies conducted on clinical strains of *Enterococci spp* isolated from odontogenic infections and these bacteria exhibit intrinsic and acquired resistance to many antibiotics.\(^{162,173}\)

Hence, PG could be used as an ideal vehicle for all the above intracanal medicaments on the basis of the numerous *in vitro* and *ex-vivo* experiments carried out in this study.
B. Secondary Objective- PCR assay and its comparison to Culture:

- Detection of selected micro-organisms by Culture:

  *Streptococcus spp* were detected in all the samples i.e., 100% of the endodontic samples from necrotic deciduous teeth by culture. These findings are in accordance with studies by Hegde et al, Pazelli et al, da Silva et al, Marsh SJ et al and Faria G; which found *Streptococcus spp* in 100%, 96.7%, 85%, 82% and 85% of root canals of primary teeth with necrotic pulp and periapical lesions respectively. Also, few of the older studies have also found a slightly lower prevalence of *Streptococcus spp* like Cohen et al (70%), and Tomic-Karovic K et al (76%). This might be due to the difference in technique used for culture to identify the microorganisms.

  *Enterococcus faecalis* was isolated in 19 out of 111 samples i.e., 17% of the endodontic samples from necrotic deciduous teeth by culture. This observation is in accordance with the study by Cogulu et al who detected 18% by means of culture only. This similarity is inspite of using different culture techniques for detection of *Enterococcus faecalis*. It is interesting to note that this is in primary infections of deciduous teeth whereas initially it was believed that *Enterococcus faecalis* was present only in teeth with failed endodontic treatment. Hegde et al found a relatively higher percentage of 35% and it might be due to the use of Enterococcus confirmatory agar in our study; which has a high specificity and low sensitivity hence should not be preferred for initial culture of samples.

  *Porphyromonas gingivalis* was isolated in 58 out of 111 samples i.e., 52% of the endodontic samples from necrotic deciduous teeth by culture. This is a higher percentage as compared to studies by Gomes et al who used VMGA transport media which found a relatively low percentage of 6.7%. This might be due to the use of a
different transport media and the one used in our study i.e., RTF is proven to be an efficient transport media. Other studies by da Silva et al and Pazelli et al found the percentage of black pigmented bacilli (BPB consisting of mainly the Porphyromonas spp and Prevotella spp ) to be 30% and 35.5%. The higher rate of detection might be even due to advancement in anaerobic culture techniques in the last decades.

- Detection of selected microorganisms by PCR:

  Enterococcus faecalis was isolated in 45 out of 111 samples i.e., 41% of the endodontic samples from necrotic deciduous teeth by PCR. These findings are higher when compared to the detection rate of 14% by Cogulu et al and similar to findings of Enterococcus spp of 50% by Fabris et al. Both the authors have used PCR for detection. This also highlights the importance of thorough disinfection of root canals of deciduous necrotic teeth in order to achieve successful pulp therapy as Enterococcus faecalis is closely related to failure of pulp therapy.  

  Porphyromonas gingivalis was isolated in 87 out of 111 samples i.e., 78% of the endodontic samples from necrotic deciduous teeth by PCR. This is similar to results by Fabris et al (49%) and Gomes et al (100%) who detected Porphyromonas gingivalis using PCR. Though our percentage of detection is higher, it is also important to note that our detection by culture was higher too and all samples positive for P. gingivalis by culture were also positive by PCR. Cogulu et al detected 16% of P. gingivalis by PCR and these findings are contrary to our findings.

  Treponema denticola was detected in 79 out of 111 samples i.e., 71% of the endodontic samples from necrotic deciduous teeth by PCR. These findings are contrary to the findings of Cogulu et al which detected a low i.e., 16% of Treponema
denticola by PCR where as Gomes et al\textsuperscript{109} detected 40% of *Treponema denticola* in the root canal samples from necrotic deciduous teeth by means of PCR.

- **Comparison of Detection of selected microorganisms by Culture and PCR:**

  *Enterococcus faecalis* and *Porphyromonas gingivalis* were detected by both culture and PCR. *Enterococcus faecalis* was isolated in 17% of the endodontic samples from necrotic deciduous by culture and 41% by PCR. *Porphyromonas gingivalis* was isolated in 52% of the endodontic samples from necrotic deciduous teeth by culture and 78% by PCR. The difference between the detection rates by culture and PCR for both *Enterococcus faecalis* and *Porphyromonas gingivalis* was statistically significant (p < 0.001, Mc Nemar test). This is contrary to the findings of Cogulu et al as they did not find the difference between detection by culture and PCR to be statistically significant and this is the only study comparing culture and PCR for detection of specific microorganisms in deciduous teeth. Many studies in permanent dentition already agree upon the fact that PCR is a more rapid, sensitive and specific technique for detection of specific microorganisms.\textsuperscript{176}
SUMMARY

The present research was conducted to assess the antimicrobial activity of various combinations of intracanal medicaments and vehicles on selected pathogens. The microorganisms were *Streptococcus spp*, *Porphyromonas gingivalis*, and *Enterococcus faecalis* isolated from root canals of deciduous molars with necrotic pulp in 5-8 old children attending the Department of Pedodontics and Preventive Dentistry, KLE VK Institute of Dental Sciences, Belagavi.

This experimental study was conducted in 2 phases. The Phase 1 involved three *in vitro* studies against selected American Type Culture Strains (ATCC) strains. The first *in vitro* study assessed the bactericidal activity of vehicles using broth dilution method. This was followed by assessment of antibacterial activity of intracanal medicaments and different vehicle combinations using agar well diffusion using pure and commercial drugs. The Phase 2 consisted of a pilot study followed by the main study which comprised of two parts related to the primary and secondary objectives. The primary objective of the research consisted of assessment of the antibacterial activity of the three intracanal medicaments, i.e. DAP, modified-DAP and Chlorhexidine with the three vehicles, namely Glycerine, PEG and PG against three selected endodontic microorganisms using an ex–vivo model. The selected endodontic pathogens (*Streptococcus spp*, *Enterococcus faecalis*, *Porphyromonas gingivalis*) were isolated from deciduous molars with necrotic pulp.

The same endodontic sample after DNA extraction was used for the secondary objective of research i.e., PCR assay for detection of the specified organisms i.e. *Enterococcus faecalis*, *Porphyromonas gingivalis* and *Treponema denticola*. Conventional PCR was used for detection of *Enterococcus faecalis* and it was compared with detection by culture. Multiplex PCR was done for detection of
Porphyromonas gingivalis and Treponema denticola; and detection of Porphyromonas gingivalis only was compared to its detection by culture too.

The intracanal medicaments, vehicles and ATCC strains were procured as pure drugs were required for the Phase 1 in vitro studies. The first in vitro study for evaluating bactericidal activity of vehicles by broth dilution assessed PG, Glycerine, PEG 400, PEG 1000 and combination of PG + PEG 400 on ATCC strains. The second in vitro study for evaluating antimicrobial activity of intracanal medicaments and vehicle combinations by agar well diffusion utilized DAP (C+M), modified DAP (A+M) and 2% CHX using pure drugs. The third and the last in vitro study of Phase 1 evaluating antimicrobial activity of intracanal medicaments and vehicle combinations by agar well diffusion utilized commercial drugs of the same medicaments. The vehicles used in both the agar well diffusion studies were PG, Glycerine, PEG 400 and PG+PEG 400 on selected ATCC strains.

Prior to the ex-vivo study the relevant permissions were obtained. A total of one hundred eleven endodontic(111) samples were taken from 37 teeth (three root canals per tooth) from 35 (15 female and 20 male) 5-8 year old children residing in Belagavi city and attending the outpatient department of Pedodontics and Preventive Dentistry, Belagavi, India. Following standardization, disinfection and isolation of selected deciduous teeth was done and endodontic samples collected in RTF. These samples were streaked on Blood agar, Kanamycin blood agar, Enterococcus Confirmatory Agar and incubated for 37 °C for 48 hrs and 72 hrs respectively in anaerobic and candle jar as per the requirements of the organism to be grown and detected. The CFUs were counted and pure colonies were picked and gram staining was done in order to confirm the microorganism. Antimicrobial susceptibility was
carried out against the specified, isolated and confirmed organisms using *ex-vivo* model. Antimicrobial susceptibility was assessed for a total of nine groups namely; C+M+G, A+M+G, CHX+G, C+M+PEG, A+M+PEG, CHX+PEG, C+M+PG, A+M+PG and CHX+PG against each of the isolated strains of *Streptococcus spp*, *P. gingivalis* and *E. faecalis*.

DNA isolation was done of the same endodontic sample after culturing and assessed by Conventional PCR for *E. faecalis* and Multiplex PCR for *P. gingivalis* and *Treponema denticola*. Detection of *E. faecalis* and *P. gingivalis* was compared to it’s detection by culture respectively. Data was analyzed using SPSS software version 22. The level of significance was set at p < 0.05. Kruskal Wallis test, Mann Whitney ‘U’ test, Shapiro-Wilk test, and McNemar test were used for data analysis, wherever applicable.

The first *in vitro* study of Phase 1 revealed that all the tested vehicles exhibited bactericidal activity though at different concentrations on the ATCC strains of *Staphylococcus aureus*, *S. mutans*, *E. faecalis* and *E. coli*. PG or propylene glycol showed bactericidal activity against *S. mutans*, *E. coli* and *E. faecalis*; which is commonly associated with root canal treatment failure was terminated at a very low concentration of 25%. PEG 1000 exhibited bactericidal activity against *S. mutans* and *E. coli* at the lowest concentration of 25%. Glycerine and Combination of Propylene glycol and PEG 400 were the vehicles with least bactericidal activity against selected pathogens.

The second *in vitro* study of Phase 1 evaluated MIC of single pure form of endodontic medicaments only and all organisms were resistant to Metronidazole. Later MIC of Ciprofloxacin with Metronidazole against *S. mutans*, *E. faecalis* and *E.
coli was 1.95µg/ml; where as against S. aureus and P. gingivalis was 7.81µg/ml and 0.039µg/ml respectively. MIC of Amoxicillin clavulanate with Metronidazole against S. mutans and P. gingivalis was 3.90µg/ml and 0.019µg/ml respectively. MIC of Amoxicillin clavulanate with Metronidazole against S. aureus, E. faecalis and E. coli was 15.625µg/ml. The zones of inhibition of all of the selected organisms were recorded to measure their antibacterial activity using agar well diffusion method.

Amongst the mean zones of inhibition for S. mutans, the highest was for CHX+PG (32.00±1.00); for S. aureus, the highest was for CHX+PEG (39.00±1.00); for E. faecalis, the highest was for CHX+PG (31.67±1.52); and for P. gingivalis, the highest was for CHX+Glycerine (27.67±1.52). Hence in pure forms 2% CHX was most effective with PG against ATCC strains of S. mutans and E. faecalis.

The third and final in vitro study of Phase 1 evaluated the antibacterial activity of commercial preparations of endodontic medicament with above mentioned vehicles in terms of zones of inhibition of all of the selected organisms using agar well diffusion method. A+M with all the four vehicles against S. mutans exhibited maximum zone of inhibition with A+M+PG (33.00±0.00). Similarly, the highest zone of inhibition for S. aureus was of A+M+Glycerine (33.67±0.577), for E. faecalis was of A+M+PG (31.33±0.577) and for E. coli was of A+M+PG(32.33±1.528). Overall observations can be summarized as Chlorhexidine is the least effective while A+M is the most effective intracanal medicament. Of all the vehicles, PG is the most effective vehicle when used in combination with intracanal medicaments.

The main study of Phase 2 initially evaluated the organisms detected and their association with signs and symptoms present in subjects. There was no significant relation between any of the organisms detected and the signs and symptoms present including radiolucency detected in furcation or periapical area radiographically.
During evaluation of parameters related to primary objective, comparison of antimicrobial activity in terms of zones of inhibition (mms) of all nine groups of medicaments and vehicle combinations against *Streptococcus* spp, *Porphyromonas gingivalis* and *Enterococcus faecalis* was done. There was statistically significant difference (p<0.001) between the nine groups against *Streptococcus* spp and the maximum zone of inhibition was of A+M+PG was 32.26 ±7.77. There was statistically significant difference (p<0.001) between the nine groups against *Porphyromonas gingivalis* and the maximum zone of inhibition was of A+M+PG was 39.37 ±9.27. There was statistically significant difference (p<0.001) between the nine groups against *Enterococcus faecalis* the maximum zone of inhibition was of A+M+PEG was 34.53 ± 7.34.

To know exactly which intracanal medicament and vehicle were effective, comparisons were done between all three medicaments keeping vehicles constant and all three vehicles keeping medicaments constant. This would give the idea of the effectiveness of each medicament and vehicle individually and which of the two to combine for maximum bactericidal effect and thus, effective disinfection of the root canals of deciduous necrotic molars. The difference between antimicrobial activity of all the three medicaments with each of the three vehicles as vehicle against selected microorganisms was statistically significant.

Following are the intracanal medicament and vehicle combinations using same vehicle but having statistically significant differences in antimicrobial activity or their zones of inhibition. For *Streptococcus* spp, A+M+G (31.32 ± 6.85) had greater antimicrobial activity as compared to C+M+G (28.75 ± 7.11) (p=0.009), A+M+PEG (31.95 ± 7.13) had greater antimicrobial activity as compared to C+M+PEG (26.03 ± 8.36) (p=0.001), A+M+PG (32.26 ± 7.77) had greater antimicrobial activity as
compared to C+M+PG (28.60 ± 7.82) (p=0.009).

For *P. gingivalis*, A+M+G (35.75 ± 9.28) had greater antimicrobial activity as compared to C+M+G (29.67 ± 8.41) (p=0.02), A+M+PEG (35.25 ± 7.61) had greater antimicrobial activity as compared to C+M+PEG (29.75 ± 7.39) (p=0.002), A+M+PG (39.37 ± 9.27) had greater antimicrobial activity as compared to C+M+PG (30.42 ± 7.01) (p<0.001).

For *E. faecalis*, A+M+G (33.58 ± 7.24) had greater antimicrobial activity as compared to C+M+G (30.21 ± 7.25) (p=0.15), A+M+PEG (34.53 ± 7.34) had greater antimicrobial activity as compared to C+M+PEG (28.47±6.96) and the difference was significant (p=0.007), A+M+PG (33.79 ± 8.54) had greater antimicrobial activity as compared to C+M+PG (31.11 ± 10.66) (p=0.39).

The difference between antimicrobial activity of all the three vehicles with each of the two medicaments i.e., C+M and A+M against *Streptococcus spp* and *Porphyromonas gingivalis* was statistically significant.

For *Streptococcus spp*, C+M+G (28.75 ± 7.11) had greater antimicrobial activity as compared to C+M+PEG (26.03 ± 8.36) (p=0.02). C+M+G (28.75 ± 7.11) and C+M+PG (28.60 ± 7.82) had similar antimicrobial activity (p=0.87). C+M+PG (28.60 ± 7.82) had greater antimicrobial activity than C+M+PEG (26.03 ± 8.36) (p=0.04).

For *P. gingivalis*, A+M+G (35.75 ± 9.28) had greater antimicrobial activity as compared to A+M+PEG (35.25 ± 7.61) and the difference was not significant (p=0.94). A+M+G (35.75 ± 9.28) had lesser antimicrobial activity to A+M+PG (39.37 ± 9.27) and the difference was not significant (p=0.06). A+M+PG (39.37 ± 9.27) had greater antimicrobial activity than A+M+PEG (35.25 ± 7.61) and the difference was statistically significant (p=0.009).
The mean zones of inhibition in mms of all the three medicaments with all the three vehicles against *E. faecalis* were not significantly better, for eg CHX+G was 26.58 ± 6.31, CHX+PEG was 25.68 ± 7.17, CHX+PG was 25.16 ± 8.46 and the difference between them was not significant (p=0.79). The clinical strains might have developed resistance to bactericidal activity of vehicles in comparison to the ATCC strains used for the *in vitro* study of Phase 1A.

Overall to summarize the findings of this study, modified DAP (A+M) is the most effective intracanal medicament and can replace the currently utilized DAP (C+M) for clinical purposes. The possibility of CHX being explored further as at times there was no significant difference in its antimicrobial activity as compared to DAP (C+M) especially against virulent microbial species like *Enterococcus faecalis*. Among the vehicles studied in this research work, Propylene glycol(PG) shows promising results consistently during the *in vitro* and *ex-vivo* studies; with all the medicaments and is recommended to be utilized to dispense intracanal medicaments.

*Streptococcus spp* were detected in 100% of the endodontic samples from necrotic deciduous teeth by culture alone. *Treponema denticola* was detected in 63% of the endodontic samples from necrotic deciduous teeth by PCR alone.

*Enterococcus faecalis* was isolated in 18% of the endodontic samples from necrotic deciduous by culture and 41% by PCR and the difference in detection was statistically significant (p<0.001; McNemar test). *Porphyromonas gingivalis* was isolated in 58% of the endodontic samples from necrotic deciduous teeth by culture and 76% by PCR and the difference in detection was statistically significant (p<0.001; McNemar test). Hence, for detection of microbiota in deciduous root canals, PCR is a better detection tool in comparison to culture for *Enterococcus faecalis* and *Porphyromonas gingivalis*, the difference being statistically significant (p<0.001).
CONCLUSION

The present study showed that the Modified Double Antibiotic Paste (A+M) was more effective than the currently recommended Double Antibiotic Paste (C+M). Hence, A+M consisting of Amoxycillin clavulanate with Metronidazole can be used as an intracanal medicament for effective disinfection of root canals prior to root canal filling, revascularization procedures, non-surgical treatment of chronic periapical lesions to name a few. Also, Chlorhexidine (CHX) was effective in disinfection of root canals but had lower antimicrobial activity in comparison to the antibiotic-based intracanal medicaments. But we still believe that Chlorhexidine has a potential antimicrobial activity which should be utilized as it being a antimicrobial biocide, has the inherent advantage of not developing resistance. It also exhibits the property of substantivity which when combined with vehicles can lead to the slow, longer release and appropriate diffusion of the intracanal medicaments. As per the findings of this study, A+M is the most effective intracanal medicament with possibility of CHX being explored further.

Among the vehicles studied in this research work, Propylene glycol (PG) shows promising results consistently; with all the medicaments and is recommended to be utilized to dispense intracanal medicaments. Other vehicles also exhibited bactericidal activity and can be used to carry intracanal medicaments. These vehicles sometimes have a synergistic action while at other times are compatible with only a few medicaments. Hence, PG should be used as the ideal vehicle to carry intracanal medicament for clinical purposes like disinfection, revascularization etc. Vehicles to dispense intracanal medicaments not only, improve handling characteristics but aid in placement, diffusion and sustained release of intracanal medicaments. Thus, vehicles
should be considered an equally important constituent of intracanal medicament pastes.

It was noticed that *Enterococcus faecalis*, *Porphyromonas gingivalis* and *Treponema denticola* were detected in higher percentages by PCR i.e. 41%, 78% and 71% respectively; as compared to previous studies. Thus, it emphasizes the role of intracanal medicaments to kill these organisms prevalent in root canals for the success of pulp therapy in deciduous necrotic teeth.

Also, for detection of microbiota in deciduous root canals, PCR is a better detection tool in comparison to culture, the difference being statistically significant (p<0.001). It also aids in more rapid, sensitive and specific detection of microorganisms.

The present study had certain limitations. Formulations of Chlorhexidine could have been studied and the one with better bactericidal activity could have been selected. Also, higher percentage of CHX gel could have been prepared for greater antibacterial activity as its antibacterial activity is concentration dependant. Also, when zones of inhibition of 2% CHX gel were compared to that of 2% CHX irrigating solution, zones of inhibition of the gels were smaller and this may be attributed to the viscous nature of the gels which hamper the diffusion of CHX.

Further studies using better formulations of Chlorhexidine as an intracanal medicament as compared to antibiotics could be tried out with better polymers to improve the efficacy of Chlorhexidine gel. As in a recent study, gels loaded with antibiotics namely modified triple antibiotic paste consisting of (ciprofloxacin, metronidazole and clindamycin) and DAP (ciprofloxacin and metronidazole) were prepared at a concentration of 1mg/mL. Similarly modified DAP consisting of Amoxycillin clavulanate gels should be studied in comparison to Chlorhexidine gel.
Once *ex-vivo* studies are done, clinical trials can be undertaken. Also, this study suggests the antimicrobial activity against commonly isolated organisms from endodontic infections and would help in choosing the prescription of systemic antibiotics judiciously.


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ANNEXURE – I – INSTITUTIONAL RESEARCH AND ETHICAL COMMITTEE APPROVAL

Research and Ethical Committee
K.L.E. University’s
V.K. INSTITUTE OF DENTAL SCIENCES
Nehru Nagar, Belgaum - 590 010. Karnataka State

CERTIFICATE

This is to Certify that the synopsis titled

EX-VIVO EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF
VARIOUS COMBINATIONS OF INTRA CANAL MEDICAMENTS

VEHICLE ON SELECTED PATHOGENS FROM BACTERIUS
MOLARS WITH NECROTIC PULP submitted by
Dr. Trivedi Mohan Nadwade Ph.d. Scholar
P.G. Student

Staff, Department of PROFESSIONAL SUGICAL DENTISTRY

has been critically evaluated and has been granted ethical clearance to continue / publish the above mentioned study.

Date: 25-01-2014

PRINCIPAL
K.L.E. VK
Institute of Dental sciences, Belgaum

CO - ORDINATOR
Research Ethical Committee
K.L.E. VK Institute of Dental sciences, Belgaum
ANNEXURE-II

Ph.D. HUMAN ETHICAL COMMITTEE APPROVAL

KLE UNIVERSITY
(Formerly known as KLE Academy of Higher Education & Research, Belgaum)
(Declared as deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Government of India Notification No F.9-10/2000-U.3(A))
Office of the Registrar, KLE University,
JNMC Campus, Nehru Nagar, Belgaum-590 010, Karnataka State, India
Tel: 0831-2444444/2493779 FAX: 0831-2493777 Web: http://www.kleuniversity.edu.in E-mail: info@kleuniversity.edu.in

Ref.No.KLEU/Ethic/14-15/ D–73
2.6th May 2014

To,
Dr. Triveni Mohan Nalawade
Ph.D.Scholar,2013-14
K.L.E. University,
Belgaum.

Dear Research Scholar

The KLE University Ethics Committee on Human Subjects for Ph. D Research Project met on 29th April 2014 to consider your application for approval of the research project “EX-VIVO EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF VARIOUS COMBINATIONS OF INTRACANAL MEDICAMENTS AND VEHICLES ON SELECTED PATHOGENS FROM DECIDUOUS MOLARS WITH NECROTIC PULP”

After review of the documents submitted by you and satisfactory explanations provided to the members, the committee has provided approval for this research project.

You are requested to report to Ethical Committee of the following:
1. Any deviation from or change of the protocol.
2. Any changes in study documents.

(Dr. Hema Dhunale)
Member Secretary,
Ph.D. Ethical Committee(Human),
K.L.E. University, Belgaum

(Dr. Sudha A. Raddi)
Chairman
Ph.D. Ethical Committee(Human),
K.L.E. University, Belgaum

CC to: - The Director Academic Affairs, KLE University
- The Director Research Foundation, KLE University
- The Registrar, KLE University
- Special Officer to Hon. Vice Chancellor, KLE University, Belgaum

BSRC/2014/109
ANNEXURE - III

INFORMED CONSENT FORM

Title of the Study:

Ex-Vivo Evaluation of the Antimicrobial Activity of Various Combinations of Intracanal Medicaments and Vehicles On Selected Pathogens from Deciduous Molars with Necrotic Pulp.

Objective/Purpose of the Study:

You are being invited to participate in this study, to learn about the germs in the teeth and the most effective method to eliminate them. The participants in the age group of 5 to 8 years coming to KLE VK Institute of Dental Sciences with the complaint of tooth problem due to decay in teeth; fulfilling the inclusion and exclusion criteria and agree to participate will be included in the study. This study will help us to treat the teeth in a better way, and minimize failures in treatment. The principal investigator is Dr. Triveni Mohan Nalawade, Ph.D. Research Scholar. This project is not funded by any organization.

Explanation of Procedure:

After taking your approval, the tooth will be cleaned from within after administrating injection for making the tooth numb and applying a rubber sheet on the tooth. The pus and infected material from tooth will be collected using paper rolls. This will take about 25-30 minutes. The tooth will be treated with suitable medicament and capped appropriately one week post-operatively as per the routine treatment protocol. The pus and infected material collected from the tooth will be studied to identify predominant microbes. Further various medicines will be tested on these microbes to note the most effective medicine in destroying the microorganisms.

Risks & Benefits:

The Investigator will not promise or guarantee that you will receive any direct benefit being part of the study. Sometimes the tooth can continue to cause discomfort even after treatment. In such cases alternative treatment will be provided. The treatment of such failed teeth will be as per the decision taken by the patient’s parent.

Withdrawal:

Participation in this study is voluntary. If you don’t wish to participate in this study, you will not lose any benefits to which you are entitled. You are free to withdraw your consent and to discontinue participation in this study at any time.
Privacy and Confidentiality:

Photographing or video recording of the operation or procedures to be performed including appropriate portions of my child’s body, for medical, scientific or educational purposes provided his/her identity is not revealed by the pictures or by the descriptive texts accompanying them. Confidentiality of records identifying the subject will be coded and maintained including those who will have access to the patient’s medical records and tissue or sample processing.

Financial Incentives for participation:

The cost of the study will be borne by the Researcher. There will be no payment to you for participating in this study.

Authorization to publish the results:

The results of the study will be used for teaching, medical publications and presentations. However, the participants’ identification will be kept confidential.

Questions:

If you have any questions about this study, please call Dr Triveni Mohan Nalawade, Principal Investigator at 8495000895 or Dr Sudha Raddi, Chairman, Human Ethical Committee, KLE University, Belgaum, Phone No. 0831-2444444.

Consent statement: I am making a voluntary decision whether or not to participate in this study. My signature below indicates that I have decided to participate, and I have read (or been read) the information provided above and I was given the opportunity to ask questions and that they have been answered to my satisfaction and that I have received a copy of this signed consent form.

____________________________________  __________________________________
Parent’s/ Guardian’s Name  Parent’s/ Guardian’s Signature/ left thumb print

____________________________________  __________________________________
Investigator’s Name  Investigator’s Signature

____________________________________  __________________________________
Name of the Witness  Signature of the Witness

Date: __________________________
Annexure – III – Informed Consent Form


document content in Kannada script
Annexure – III – Informed Consent Form

Informed Consent Form

1. Details of the patient's medical condition and previous treatments

2. Explanation of the proposed treatment

3. Potential risks and complications of the proposed treatment

4. Patient's understanding of the proposed treatment

5. Consent to proceed with the treatment

6. Signature of the patient or their legal representative

7. Date of consent

8. Contact information for further queries
ನೂಗು :–

ಅ ಪ್ರತ್ಯೇಕವಾಗಿ ಮೂಲಕ ಮುಂದೆ ಪ್ರತ್ಯೇಕ ಸೇರಿಸಿದ್ದಾಗ ಅವರು ಆಧುನಿಕ 849500895 ಎನ್ನುವ ಅವರ ಮುಂದೆ ಕೆಲಸ (ಮುನ್ನಾದ) ಸಂಪರ್ಕ ಪಟ್ಟೆ (0831-2444444) ಅಂತಿಹಾದ ಸಂಪರ್ಕ ಪಟ್ಟೆಯನ್ನು ಅಳಿಸಿ ಮಾಡಬೇಕು. ಆದರೆ ಆರೋಗ್ಯ ಪ್ರತ್ಯಾಮಕಾರಿಯರ ಪ್ರತ್ಯೇಕವಾಗಿ ಮುಂದೆ ಸೇರಿಸಿದ್ದಾಗ ಅವರು ಆಧುನಿಕ 849500895 ಎನ್ನುವ ಅವರ ಮುಂದೆ ಕೆಲಸ (ಮುನ್ನಾದ) ಸಂಪರ್ಕ ಪಟ್ಟೆ (0831-2444444) ಅಂತಿಹಾದ ಸಂಪರ್ಕ ಪಟ್ಟೆಯನ್ನು ಅಳಿಸಿ ಮಾಡಬೇಕು.

ನೂಗುವಿನ ಸೇರಿಸಿ:–

ಕೊನೆಯು ಸೇರಿಸಿದ್ದಾಗ ಅ ಪ್ರತ್ಯೇಕವಾಗಿ ಪ್ರತ್ಯೇಕವಾಗಿ ಸೇರಿಸಿದ್ದಾಗ ಅವರು ಆಧುನಿಕ 849500895 ಎನ್ನುವ ಅವರ ಮುಂದೆ ಕೆಲಸ (ಮುನ್ನಾದ) ಸಂಪರ್ಕ ಪಟ್ಟೆ (0831-2444444) ಅಂತಿಹಾದ ಸಂಪರ್ಕ ಪಟ್ಟೆಯನ್ನು ಅಳಿಸಿ ಮಾಡಬೇಕು. ಅಂತಿಹಾದ ಸಂಪರ್ಕ ಪಟ್ಟೆಯನ್ನು ಅಳಿಸಿ ಮಾಡಲು ನಗರಾಧಿಕಾರಿಯರ ಪ್ರತ್ಯೇಕವಾಗಿ ಸೇರಿಸಿದ್ದಾಗ ಅವರು ಆಧುನಿಕ 849500895 ಎನ್ನುವ ಅವರ ಮುಂದೆ ಕೆಲಸ (ಮುನ್ನಾದ) ಸಂಪರ್ಕ ಪಟ್ಟೆ (0831-2444444) ಅಂತಿಹಾದ ಸಂಪರ್ಕ ಪಟ್ಟೆಯನ್ನು ಅಳಿಸಿ ಮಾಡಬೇಕು.

_____________          ______________
ನಗರಾಧಿಕಾರಿ/ಅಧಿಪತಿ ಪ್ರತ್ಯೇಕವಾಗಿ          ನಗರಾಧಿಕಾರಿ/ಅಧಿಪತಿ ಪ್ರತ್ಯೇಕವಾಗಿ

_____________          ______________
ನಗರಾಧಿಕಾರಿ/ಅಧಿಪತಿ ಪ್ರತ್ಯೇಕವಾಗಿ          ನಗರಾಧಿಕಾರಿ/ಅಧಿಪತಿ ಪ್ರತ್ಯೇಕವಾಗಿ

_____________          ______________
ನಗರಾಧಿಕಾರಿ/ಅಧಿಪತಿ ಪ್ರತ್ಯೇಕವಾಗಿ          ನಗರಾಧಿಕಾರಿ/ಅಧಿಪತಿ ಪ್ರತ್ಯೇಕವಾಗಿ

ನಿಂದಿನ:
Annexure – III – Informed Consent Form

संस्थी पत्र

शीर्षक :- "एक्स–व्यायाम विज्ञापन ऑफ द अंडरी मायोरियल ऑटोस्टिकट्री ऑफ
बेट्रियस कॉल्बीनेशन्स ऑफ इंटरनॅशनल मेडिकमेन्डस ऑफ वेबिकल्स ऑफ सिंगलकटेड
पेढोजेन्स फ्रॉम डेसिडियस मोलार्स विथ नेक्रोटिक पत्थर."

संस्थानवाचे कारण : तुम्ही हा संस्थानात सहभाग घेण्यासाठी स्वागत करत आहेत. हे संस्थान
दातानमध्ये सापडणारे जंतु आणि त्यांना नाही करण्याचे सर्वांगी आरोप आहेत.
सहभाग घेणारी मुळे के.एल.वि.के.इन्स्टीट्यूट ऑफ डेंटल सायंसेस ला दाताची किंवा
दातान संदर्भता तकार घेऊन घेऊने ५ ते ८ वर्षातील असतील. हे पॅकेज स गणव्या
नियमांकनाचे व त्यांचा मजीने निवडिलेले असतील. हा संस्थानाचे दाताच्या उपचारप्रती
मध्ये सुधारणा होईल व अपवाचे प्रमाण कमी घेंबरे. हा संस्थानाची प्रमुख सुरूवातक
डॅं त्रिवेणी मोहन नलावडे आहेत. हा संस्थानाला कुटस्थायी आयोजककंडून आर्थिक
सहाय्यांत नाहीत.

प्रकटपूर्व: स्मरणीकरण : तुम्ही संस्थी पेटेल्यांतः, दाताला करण त्यांचा सर्व जीत
लावण्यात घेंबरे. त्यांतरं दाताच्या साफ करण्यात घेंबरे व दातातला पू व जंतू, कागदाच्या
सूचीचा पोशून घेण्यात घेंबरे. त्यांतरं दाताची योग औषधांचे उपचार करणे नियमांकनांची एक
आठवड्या नंतर कंप वस्तवी जाईल.

शास्त्री पृष्ठभूमी व फायदे : संस्थानात भाग घेण्यासाठी, कुटस्थायी प्रकारच्या
आर्थिक फायदा किंवा मुनास्वव्हादह आश्वासन किंवा खासी देत नाही. कमी कमी दाताच्या
उपचारांतरं त्रास किंवा अस्वास्थ्य होऊ शकते. अश्यावेळी दूरस्थी उपचारप्रकार वापरण्यात
घेंबरे. अश्या दाताच्या उपचारात बहुत निर्यात रुपांचे (पैसेंटची) पायलट घेंबरे.
Annexure – III – Informed Consent Form

संस्थापनातून माही घेण्यासाठी कुटल्याही प्रकारच्या द्रवण किवा जबरदस्ती नाही, जर तुम्हाला, हा संस्थापनात सहभाग घेण्याची इच्छा नाही, तरीही तुमची उपचारसेवा बंद करण्यात येणार नाही. तुम्हाला संस्थापनाच्या दिलेली सांभवी, कुटल्याही वेळी पडते प्रेरणाचे स्वातंत्र्य आहे आणि तुम्ही कुटल्याही वेळी संस्थापनातून माही घेण्याचे नकारात.

खाजगीत व तुम्ही :- प्रज्ञानिक आणि शेषकाणक कारणासाठी उपचार करताना त्याचे प्रोटोकॉल किवा अध्यापणिक कार्यक्षमता येतील या आवश्यक आहे. पूर्णता गोपीच्या शेवटचा ठेवला येईल. पूर्णता सर्वांच्या कार्याबंधन व दातात येतल्या नुसरे गोपीच्या ठेवला येतील.

आर्थिक लाभांश :- संस्थापनाचा सांपूर्ण खर्च प्रमुख संस्थापकाचे कर्त्य सहील व तुम्हाला संस्थापनात भाग घेण्यासाठी कुटल्याही प्रकारचे आर्थिक लाभ भेटणार नाही.

निवडल प्रकाशित कार्याची आयोजने:

हा संस्थापनाचे निवडल (निवड) आयोजने, नैदिकीय प्रकाशन व साहित्यी कार्याची वापरण्यात येतील. यादर्श नामांकन घेण्याचे व्यावसायिक आंदोलन गोपीच्या ठेवल्यात येईल.

प्रश्न (शंकास) :- जर तुम्हाला संस्थापन बदल कुटल्याही प्रकारचे प्रश्न किवा शंका असतील तर तुम्ही व त्याच्या प्रश्नात वर्तील शंका, प्रमुख संस्थापक झांस या शंका असतील. संस्थापणाची नैसर्गिक सांभवी झांस या शंका असतील, झांस या शंका म्हणजेच मान्य करू शकता.

सांभवी विधान :- ही हा संस्थापनात सहभाग घेण्याची किंवा न घेण्याची निर्णय विनामूल्य आणि स्वत्यूळांच्या मोडाच्या प्रकारच्या दराचाच्या नेत्रे नेत्रे; ही अनुसूचित (पदवींची) देत आहे.
माइया खालील स्वास्थी (सही) दर्जन आहे कि मी संगोष्ठन संरचनित सरग्रही माहिती वाचून आपण माइया प्रश्नांची समाधानकारक उत्तरे दिल्यानंतर, हा संगोष्ठन संरचनात सहभाग पेटेन. मला स्वास्थी संस्थानी पत्रांनी एक प्रत देण्यात आली आहे:

पालकांचे नाव:

पालकांची स्वास्थी/अंगाठा

डॉक्टरांचे/संशोधकांचे नाव:

डॉक्टरांचे/संशोधकांचे स्वास्थी

साक्षीदाचे नाव:

साक्षीदाची स्वास्थी/सही

तारीख/दिनांक:
ANNEXURE – IV - ASSENT FORM
DEPT. OF PEDODONTICS AND PREVENTIVE DENTISTRY
K.L.E.V.K. INSTITUTE OF DENTAL SCIENCES.
BELGAUM.

My name is Dr Triveni Mohan Nalawade. I am a dentist. I am doing a study to learn about the germs in your teeth and how to destroy them. If you agree to be in my study, you will be asked to help me in cleaning your teeth with a shower after putting your tooth to sleep and let me take out germs using a small paper roll.

You can ask questions at any time that you might have about this study. Also, if you decide at any time not to finish, you may stop whenever you want. Signing this paper means that you have read this or had it read to you and that you want to be in the study. If you don’t want to be in the study, don’t sign the paper. Your parent(s) know that I am asking you to do these things. Remember, being in the study is up to you, and no one will be angry if you don’t sign this paper or even if you change your mind later.

Signature of participant ____________________ Date __________

Signature of investigator ____________________ Date __________
Annexure – IV – Assent Form
लद्हान मुलांचे संगती पत्र

शीर्षक :- "एक्स-च्या वहिल्युएशन ऑफ द अंतरी मायकॉर्पोरेशन ऑफ इंटरनाशनल मेडिकेमेंट्स अंदून वेटिकल्स ऑफ सिलेक्टेड पॉर्टोफोर्म डेसिफ्लेक्चर मोलार्स विथ नेफ्रोटिक प्लेन."

माझे नाव डॉ. त्रिवेणी गोळ्याचल नलावडे आहे. मी लद्हान मुलांच्या दातांनी डॉक्टर आहे. मी दाताला कीड लावणार्या कीडाणु त त्यांचा नाश करण्याचा आह्वान करत आहे. जर तुमच्या अभ्यासक्रमाच्या मदत करण्यासाठी तैलार्य आहेस तर तुड्डा दाताला झोपेचे औषध देऊन, त्याच्यावर स्वाभाविक शीट लावून त्याला साफ करण्यात येईल. तुम मला छोडणार कागदाच्या सुरुवातीले कीटाणु काळ्यावर मदत करणार आहेस.

तू भा अभ्यासक्रमाच्या दृष्टीकोन विचारको शक्तीसेह जर तुला कस्तीली प्रक्रिया शांतवाच्याची असेल, तर तू तोही निर्णय घेऊ शकतो. भा कागदात तुला करण्याचा अर्थ आहे की तू भा कागद बांधून आहेस किंवा तुला वाचून दाखविलेला आहे व तू भा अध्यासात भाग घेण्यास तैलार्य आहे.

तुड्डा पालकांना भा सामग्रीक्रमाच्या माहिती आहे. लक्षात ठेव, भा अध्यासात भाग घेणे तुड्ड्याच्यात आहे, आणि तू जर भा कागदावर सही नाही केली किंवा नंतर मग बदलले तरीही तुड्ड्याच्या कोणीही समविकार नाही.

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<th>भाग घेणार्याची सही</th>
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<th>तारीख/दिनांक</th>
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</table>
ANNEXURE-V  
PROFORMA  

K.L.E SOCIETY’S  
DENTAL HOSPITAL AND RESEARCH CENTRE,  
NEHRUNAGAR, BELGAUM-590010.  
DEPARTMENT OF PEDODONTICS AND PREVENTIVE DENTISTRY  

“EX-VIVO EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF  
VARIOUS COMBINATIONS OF INTRACANAL MEDICAMENTS AND  
VEHICLES ON SELECTED PATHOGENS FROM DECIDUOUS MOLARS  
WITH NECROTIC PULP.”  

Date –  

PATIENT INFORMATION:  

NAME:  
SEX:  
AGE:  
PARENT/GUARDIAN:  

HISTORY:  

CHIEF COMPAINT:  
HISTORY OF PRESENT ILLNESS:  
RELEVANT MEDICAL HISTORY:  
PREVIOUS DENTAL HISTORY:  

NATAL HISTORY:  

POST NATAL HISTORY:  

GENERAL EXAMINATION:
INTRA-ORAL EXAMINATION

Soft Tissue Examination:

Hard Tissue Examination:

No of Teeth:

Decayed Teeth:

Filled Teeth:

Missing Teeth:

Root Stumps:

Mobility:

PROVISIONAL DIAGNOSIS:

INVESTIGATION:

INTRA ORAL PERiapICAL RADIOGRAPH

CULTURE OF BACTERIA

FINAL DIAGNOSIS:

TREATMENT PLANNING:
ANNEXURE-VI

PCR WORKSHOP CERTIFICATE

This is to certify that [Participant's Name], in the workshop on "PCR Approach in Molecular Diagnostics and Research" held on [Date] at [Location], has participated as a [Title]. The workshops were conducted by [Organizers].

[Signature]
[Name]
[Position]
[Institution]
ANNEXURE – VII – STANDARD OPERATING PROCEDURES

I] Standard Operating Procedures for Weighing Balance:

OBJECTIVE: Advanced laboratory training taken to provide a procedure for operation of Digital balance in Dr. Prabhakar Kore Basic Science Research Centre.

SCOPE: Applicable to maintenance of all weighing balances of different capacities in Dr. Prabhakar Kore Basic Science Research Centre, Belgaum.

RESPONSIBILITY: All the technicians, research assistants, research associates and Ph.D scholars working at Dr. Prabhakar Kore Basic Science Research Centre, Belgaum.

ACCOUNTABILITY:

1.0 HEALTH, SAFETY AND ENVIRONMENT: Electricity, Chemicals, Drugs & Extracts.
   i) Microbial contamination of chemicals, drugs and extracts.
   ii) Electric shocks.
   iii) Chemical contamination of body, clothing due to spillage.

2.0 Model: Digital Balance AUW 220D

3.0 PROCEDURE:
   i) Ensure that the instrument is clean and free from dust and placed in such a way that any vibrations do not affect it.
   ii) Check the spirit level provided on the top of the balance.
   iii) Switch on the mains.
   iv) Press the POWER button provided on the instrument.
   v) The instrument will do calibration CAL2, CAL1, CAL0 and the digital screen will show 0.000 g reading.
vi) Press O/T button to change the display units from 0.0000 g to 0.00 mg

vii) Place the object to be weighed, on the platform of the balance.

viii) The accurate weight will be shown on the screen.

ix) The balance gives accuracy from 1mg to 220g with \( d = 0.01g \). (0-82gm)

\( d = 0.1mg \) (0-220 mg)

x) Switch off the balance when not in use by pressing ON/OFF button present on the instrument.

4.0 CALIBRATION

i) Switch ON the mains.

ii) Switch ON the ON/OFF switch provided on the instrument.

iii) Place 50g and 100g standard calibrated weights on the platform. Calibrate the machine.

iv) If any variation of more than the standard limit is observed during calibration, weighing balance should be labeled as "OUT OF ORDER". Inform about the variance to the lab assistant. Do not use balance until all errors are rectified.

v) After rectification of error in the weighing balance, calibrate the weighing balance by repeating the same procedure and variation should fall in the standard limits.

vi) All the balances should be serviced by authorized personnel on a quarterly basis. During each servicing, the balance should be cleaned and calibrated.
II] Standard Operating Procedures for Laminar Airflow Unit:

**PURPOSE:** To lay down the procedure for Operation of Laminar Airflow Unit.

**OBJECTIVE:** To provide a procedure for operating Laminar Air Flow used to maintain the area for microbial analysis in Dr. Prabhakar Kore Basic Science Research Centre.

**SCOPE:** Applicable to maintenance of all LAF units of different capacities in Dr. Prabhakar Kore Basic Science Research Centre, Belgaum

**RESPONSIBILITY:** All the technicians, research assistants, research associates and Ph.D scholars working at Dr. Prabhakar Kore Basic Science Research Centre, Belgaum.

1.0 **HEALTH, SAFETY AND ENVIRONMENT:** Heat, Electricity, UV light, Micro-organisms, Chemicals, Damaged containers.
   
i) Microbial contamination of body, clothing.
   
ii) Burns to skin and electric shocks.
   
iii) Chemical contamination of body, clothing.
   
iv) UV damage to eyes and skin.

2.0 **Model:** LAF 0913

3.0 **Operation and cleaning of LAF:**

i) Switch “OFF” the UV light.

ii) Clean the equipment with clean lint free cloth duster and spray 70% ethanol to LAF unit and entire area of LAF room.

iii) Switch “ON” the “AIR” and visible light switch situated on the control panel on right hand side of the instrument.

iv) Now check the LAF Manometer pressure, it should be within 10-20 mm of Water Gauge.
v) Check the log record for burning hour of UV light, it should not exceed more than 2000 hours. UV tube should be replaced after specified burning period.

vi) Start the gas burner with the help of gas lighter and carry out the routine works.

vii) After completion of work, switch OFF the airflow and gas burner.

viii) Clean any remaining water or waste liquid material spilled on the laminar airflow platform properly with a dry cloth.

ix) Clean properly the working chamber from ceiling, followed by the side glass and then the platform of the chamber with sterile 70% ethanol.

x) Spray sterile 70% ethanol and switch OFF the visible light and then switch ON the UV light till to start the next operation.

xi) If the LAF is used for aseptic filtration, clean the receiver pipe tank with hot WFI.

xii) Open the outlet of solution collection tank and collect the solution in plastic crate. Close the valve and transfer the crate for solution.

4.0 Cleaning of Pre filters

i) Ensure that the LAF is switch off. Affix the tag on instrument “UNDER MAINTENANCE”.

ii) Pre filter is situated on the backside of the LAF, unscrew and then remove the pre filter from LAF.

iii) Transfer the pre filter to washing area and blow the compressed air from reverse side to blow out all the dust particles.

iv) Wash the pre filter with DM water and then with liquid detergent solution. Finally wash again with DM water to remove the detergent solution.
Remove the trapped water from the pre filter by jerking and finally rinse with 70% ethanol.

Allow to dry the pre-filter at their original place and tighten the screw properly.

**5.0 Precautions**

i) Take care to prevent any damage to the integrity of filter during cleaning. In case of observation of any damage to filters, immediately inform to your superior for further action.

ii) Instrument should be cleaned when the electrical connections of the equipment is in OFF position.

iii) Clean the laminar airflow chamber after every operation.

iv) Do not work when UV light is ON as it may cause eye damage.

v) Maintain the level of the platform by adjusting the equipment from the base with the help of glass beads in such a manner that the platform level should remain horizontally flat. There should be ups and downs in any portion of the chamber.

**6.0 Frequencies**

i) Daily for operation

ii) Fortnightly for pre filter cleaning

iii) Calibration: Yearly by external agency

**7.0 Abbreviations:**

SOP: Standard Operating Procedure

NA: Not applicable

LAF: Laminar Air Flow

DM: Dematerialized
III] Standard Operating Procedures for Anaerobic Jar:

PURPOSE: To lay down the procedure for Operation of Anaerobic Culture Jar.

OBJECTIVE: To provide a procedure of Anaerobic jar to maintain anaerobic conditions for microbial culture and growth in Dr. Prabhakar Kore Basic Science Research Centre.

SCOPE: Applicable to maintenance of all anaerobic jars of different capacities in Dr. Prabhakar Kore Basic Science Research Centre, Belgaum.

RESPONSIBILITY: All the technicians, research assistants, research associates and Ph.D scholars working at Dr. Prabhakar Kore Basic Science Research Centre, Belgaum.

1.0 HEALTH, SAFETY AND ENVIRONMENT: Heat, Micro-organisms, Chemicals, Damaged containers.
   i) Microbial contamination of body, clothing.
   ii) Burns to skin.
   iii) Chemical contamination of body, clothing.

2.0 Model: Anaerobic System Mark VI

3.0 PROCEDURE:
   i) Remove the cover and place it carefully on the bench with the catalyst sachet uppermost, to reduce the risk of its contamination.
   ii) Load the culture into the jar in their dishes or containers.
   iii) Replace the cover on the jar and secure it with bridge clamp, finger tightening the Bakelite knob.
   iv) Anaerobiosis is achieved by the formation of the hydrogen and carbon dioxide gas mixture through the following reactions:
v) Citric acid + sodium carbonate $\rightarrow$ sodium citrate + carbon dioxide

vi) Sodium borohydride $\rightarrow$ sodium metaborate + hydrogen

vii) Shut both needle valves.

viii) Open the vacuum needle valve and allow the pressure in the jar to fall to or below 10 mm mercury. The cover will tend to pull down under vacuum.

ix) Close the valve after adding the chemicals and remove both hydrogen and vacuum connections.

x) Incubate the jar at 35 degree C to 37 degree C for the required period ie 48 hrs for anaerobes.

xi) After incubation, remove the bridge clamp and open one of the needle valves so that any reduced pressure inside the jar will be released and the cover can be removed.

xii) The catalyst which is provided to catalyze the reaction between residual oxygen and added hydrogen normally remains active for long periods.

xiii) To activate the catalyst before a cycle, it should be heated in an oven for 90 minutes at 160 degree C.

xiv) Anaerobic conditions should always be monitored when using anaerobic jar technique by including an oxidation-reduction indicator. Methylene blue strips are available. Alternatively, a 13 x 100 mm test tube containing a few ml of methylene blue-NaHCO₃-glucose mixture can be placed in jar. After autoclaving fill 2/3 of the tube with the indicators. Cool and keep it in refrigerator until required.
xv) Use the indicator by placing it in the culture jar with its open end uppermost. When oxygen is absent it reverts to its colorless form, when it is constantly blue, either the catalyst is inert or the cover is leaking.

4.0 Abbreviations:

SOP – Standard Operating Procedure

% – Percent

gm – Gram

IV] Standard Operating Procedures for Bacteriological Incubator:

PURPOSE: To provide a procedure of bacteriological incubator used to incubate bacterial cultures in Dr. Prabhakar Kore Basic Science Research Centre.

OBJECTIVE: To describe the procedure for calibration of bacteriological incubator.

SCOPE: Applicable to calibration of bacteriological incubators of different capacities in Dr. Prabhakar Kore Basic Science Research Centre, Belgaum.

RESPONSIBILITY: All the technicians, research assistants, research associates and Ph.D scholars working at Dr. Prabhakar Kore Basic Science Research Centre, Belgaum.

1.0 HEALTH, SAFETY AND ENVIRONMENT: Heat, Electricity, Micro-organisms, Chemicals, Damaged containers.

   i) Microbial contamination of body, clothing.
   ii) Burns to skin and electrical shock.
   iii) Chemical contamination of body, clothing.

2.0 MODEL: BDI-54 Labotech bacteriological incubator.
3.0 PROCEDURE :

A] General cleaning procedure
   i) Ensure that the power supply to the incubator is switched OFF.
   ii) De-dust the incubator daily externally with a clean dry cloth.
   iii) Once in a week remove adhered dust by wet mopping using soap solution.
       Afterwards wipe the surface with a clean dry cloth to remove the moisture.
   iv) Mop the interior surfaces with a clean dry cloth, daily.

B] Operating procedure
   i) Ensure that the incubator is properly connected to the power supply.
   ii) Switch ‘ON’ the main switch and then the cabinet switch.
   iii) Set the required temperature to 37.0 degree C by pressing the set knob and the soft keys.
   iv) Monitor the temperature daily as per following procedure.
   v) Temperature shall be recorded which is displayed on LCD of controller of incubator.
   vi) Observe the temperature shown on digital display. The temperature should not differ by +/- 2 degree C.
   vii) Report any discrepancy observed during operation or temperature monitoring to the person incharge.
   viii) Inform to Engineering Department for rectification and put the status label of ‘Under Maintenance’.
   ix) Maintain the record of incubator usage.
   x) Fill the temperature record regularly.

4.0 ABBREVIATIONS:

SOP – Standard Operating Procedure
V] Standard Operating Procedures for Hot Air Oven:

**PURPOSE:** To provide a procedure for operation of Hot Air Oven in Dr. Prabhakar Kore Basic Science Research Centre.

**SCOPE:** Applicable to operation of weighing balances in Dr. Prabhakar Kore Basic Science Research Centre.

**RESPONSIBILITY:** All the technicians, research assistants, research associates and Ph.D scholars working at Dr. Prabhakar Kore Basic Science Research Centre.

1.0 **HEALTH, SAFETY AND ENVIRONMENT:** Heat, Electricity and Chemicals

i) Chemical contamination of body, clothing.

ii) Burns to skin and electrical shock.

2.0 **MODEL:** BDI-50 (Memmert Type)

3.0 **PROCEDURE:**

i) Ensure the cleanliness of the instrument.

ii) Open the ventilation knob provided on top of the oven.

iii) Switch “ON” the power supply.

iv) Electronic temperature controller displays the chamber temperature.

v) Set the required temperature by pushing the “PUSH” switch and first potentiometer knob clockwise or anticlockwise until the temperature comes to set one.

vi) Set the temperature with the help of second potentiometer knob.

vii) Release the “PUSH” switch.

viii) Indicator Bulb glows indicates that the power to the heater is “ON”.

ix) Switch “ON” the fan switch for air circulation.
x) Use rotary switch for precise control of temperature.

xi) Four positions of Rotary switch are available as follows:

- 0 - Off position  
- 1 - 5°C above ambient to 90°C,  
- 2 - 90°C to 150°C  
- 3 - 150°C to 250°C

xii) Keep the switch on suitable markings as per requirements of temperature.

4.0 ABBREVIATIONS:

SOP : Standard Operating Procedure  
No. : Number

VI] Standard Operating Procedures for Vortex Mixer:

PURPOSE : To define the operation of the instrument for better and error free use in Dr. Prabhakar Kore Basic Science Research Centre.

SCOPE : Applicable to mix the solution or to dissolve the various substance in to solvent in Dr. Prabhakar Kore Basic Science Research Centre.

RESPONSIBILITY: All the technicians, research assistants, research associates and Ph.D scholars working at Dr. Prabhakar Kore Basic Science Research Centre.

1.0 HEALTH, SAFETY AND ENVIRONMENT : Heat, Electricity, Micro-organisms, and Chemicals.

i) Microbial contamination of body, clothing.

ii) Burns to skin and electrical shock.

iii) Chemical contamination of body, clothing.

2.0 MODEL: RIVOTEK 50141022.

3.0 PROCEDURE :

i) Switch “ON” the power supply.

ii) Switch “ON” the instrument.
iii) Red light will glow.

iv) Put the test tube on the rubber pad.

v) Set the required speed with the help of the knob.

vi) On completion turn the speed knob anticlockwise.

vii) Switch “OFF” the instrument.

viii) Switch “OFF” the power supply.

4.0 ABBREVIATIONS: NIL
Bactericidal activity of propylene glycol, glycerine, polyethylene glycol 400, and polyethylene glycol 1000 against selected microorganisms

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Abstract

**Aim:** The aim of the present study was to evaluate the bactericidal activity of propylene glycol, glycerine, polyethylene glycol 400 (PEG 400), and polyethylene glycol 1000 (PEG 1000) against selected microorganisms in vitro. **Materials and Methods:** Five vehicles, namely propylene glycol, glycerine, PEG 400, PEG 1000, and a combination of propylene glycol with PEG 400, were tested for their bactericidal activity. The minimum bactericidal concentration was noted against four standard strains of organisms, i.e. Streptococcus mutans ATCC 25175, Streptococcus mutans ATCC 13987, Enterococcus faecalis ATCC 55550, and Escherichia coli ATCC 25922, using broth dilution assay. Successful endodontic therapy depends upon thorough disinfection of root canals. In some refractory cases, routine endodontic therapy is not sufficient, so intracanal medicaments are used for proper disinfection of canals. Intracanal medicaments are dispensed with vehicles which aid in increased diffusion through the dental tubules and improve their efficacy. Among the various vehicles used, glycerine is easily available, whereas others like propylene glycol and polyethylene glycol have to be procured from appropriate sources. Also, these vehicles, being viscous, aid in sustained release of the medicaments and improve their handling properties. The most commonly used intracanal medicaments like calcium hydroxide are ineffective in many microorganisms, while most of the other medicaments like MTAD (Mixture of Tetracycline, an Acid, and a Detergent) and Triple Antibiotic Paste (TAP) consist of antibiotics which can lead to development of antibiotic resistance among microorganisms. Thus, in order to use safer and equally effective intracanal medicaments, newer alternatives like chlorhexidine gluconate, ozonized water, etc., are being explored, and these vehicles are being tested in this study. **Results:** All vehicles exhibited bactericidal activity at 100% concentration. **Conclusion:** Propylene glycol was effective against three organisms namely S. mutans, E. faecalis and E. coli and its bactericidal activity was at 50%, 25% and 50% respectively. PEG 1000 was effective against S. mutans and E. coli at 25%. Hence propylene glycol was effective on more number of organisms of which E. faecalis is a known resistant species. PEG 1000 was bactericidal at a lower concentration but was effective on two organisms only.

**Key words:** Bactericidal activity, broth dilution, minimum bactericidal concentration, minimum inhibitory concentration, polyethylene glycol 400 and polyethylene glycol 1000, propylene glycol

INTRODUCTION

Last three decades have been very challenging, especially due to the global menace of developing antibiotic resistance.[i] Newer drugs are being discovered with millions and billions being invested in drug research.[ii] Instead, the need for developing newer alternatives or unexplored properties of existing agents

1 Journal of International Society of Preventive and Community Dentistry March-April 2015, Vol. 5, No. 2
which can enhance the activity of pre-existing drugs or antibiotics is definitely a viable option.\textsuperscript{[2]} For instance, in the field of Endodontics, both pediatric and adult intracanal medicaments are commonly used for proper disinfection of the root canals, especially in refractory cases. Proper disinfection is the mainstay for successful endodontic therapy and it is achieved through the collective effects of biomechanical preparation, irrigation, and intracanal medication.\textsuperscript{[1-9]}

Furthermore, previous studies have shown that bacteria in infected root canals and also peripalvic tissues (especially furcation area in primary molars) reside deep within dentine, cementum, and peripalvic tissues too.\textsuperscript{[2,3]} In order to reach these areas effectively, enhanced penetration is attained by means of intracanal medicaments used along with carriers or vehicles, for example, propylene glycol.\textsuperscript{[9]}

One of the properties which have been under-investigated is whether these vehicles or the so-called “excipients” in Pharmaceuticals have antimicrobial property or activity on their own. If they really possess antimicrobial activity, they can be used as an effective alternative for disinfection of root canals, with reduced probability of development of antibiotic resistance. In view of the above-mentioned facts, we planned to study the antimicrobial activity of the vehicles propylene glycol, glycercin, polylethylene glycol 400 (PEG 400), polylethylene glycol 1000 (PEG 1000), and a combination of propylene glycol and PEG 400, as this might shed some light on the ideal vehicle for intracanal medicaments. Polylethylene glycol is also referred to as “Macrogol” and the number denotes its molecular weight. Higher the number, higher is its viscosity.\textsuperscript{[10]} The combination of propylene glycol with Macrogol was first used in dentistry in vitro by Takunluge et al., whereas PEG 1000 was used in vitro recently by Carreira et al.\textsuperscript{[11-14]} To the best of our knowledge and literature search, no study of the bactericidal activity of these vehicles and their composition has been conducted till date.

MATERIALS AND METHODS

This study was carried out in Dr Prabhakar Kore Basic Science Research Centre, KLE University, Belgaum. It was approved by the Institutional Review Board of KLE University (Ref No. KLEU/Ethic/14-15/D-73) and it is a part of an ongoing ex vivo study. The susceptibility of the test organisms to propylene glycol, glycercin, PEG 400, PEG 1000, and propylene glycol with PEG 400 was assessed using broth dilution assay, as minimum inhibitory concentration (MIC) can be readily converted to the minimum bactericidal concentration (MBC). Triplicates were performed for each of the standard strains.  

- Culture media: Brain Heart Infusion (BHI) broth
- Test organisms: Four micro-organisms were selected for the study: Streptococcus mutans, American Type Culture Collection (ATCC) 25175, Staphylococcus aureus ATCC 12598, Enterococcus faecalis ATCC 35550, and Escherichia coli ATCC 25922 (Figure 1). All microorganisms were previously subcultured in appropriate media and under gaseous conditions to confirm their purity at 35°C for 48 h prior to testing of the vehicles
- Inoculum preparation: The growth method or the log phase method was performed as follows. At least three to five well-isolated colonies of the same morphological type were selected from an agar culture plate. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 4–5 ml of BHI broth. The broth culture was incubated at 35°C for 2–6 h until it achieved the turbidity of the 0.5 McFarland standard. The turbidity of actively growing broth culture was adjusted with broth to obtain a final turbidity optically comparable to that of the 0.5 McFarland standard, done visually by comparing the inoculum tube and the standard against a white card with contrasting black lines
- Broth dilution method: A total of 10 tubes were taken and nine dilutions of the vehicle were done with BHI for MIC and MBC. In the initial tube, only 200 µl of vehicle was added. For further dilutions, 200 µl of BHI broth was added to the next nine tubes separately. In the second tube,

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Standard strains against which bactericidal activity of the five vehicles was checked. (a) Streptococcus mutans ATCC 25175; (b) Staphylococcus aureus ATCC 12598; (c) Enterococcus faecalis ATCC 35550; (d) Escherichia coli ATCC 25922.}
\end{figure}
Annexure – VIII – Publications

Nalawade, et al.: Bactericidal activity of various vehicles

200 μl of vehicle was added which already contained 200 μl of BHI broth. This was considered as 10⁻¹ dilution. From the 10⁻¹ diluted tube, 200 μl was transferred to the second tube to make 10⁻² dilution. The serial dilution was repeated up to 10⁻⁸ dilution for each vehicle. From the maintained stock cultures of the required organisms, 5 μl was taken and added to 2 ml of BHI broth. In each serially diluted tube, 200 μl of the above culture suspension was added. The last tube contained only the media and the culture suspension, i.e. the growth control. The tubes were kept for incubation for 24 h at 37°C in bacteriological incubator and observed for turbidity [Figure 2].

- MBC: After recording the lowest concentration inhibiting the growth of organisms as MIC, all the tubes not showing visible growth were subcultured on BHI agar along with the control tube, i.e., growth control, and incubated overnight. The amount of growth was noted; no growth indicated the whole inoculum was killed and thus highest dilution showing 99.99% inhibition was recorded as MBC [Figure 3].

- Triplicates were performed for each of the standard strains. The experimental data were collected and statistically analyzed using Fisher’s exact test.

RESULTS

The MBC results for the vehicles are shown in Table 1. The results showed that all vehicles did exhibit bactericidal activity on the selected microorganisms at different concentrations. Out of all the vehicles, PEG 1000 was the most effective antemicrobial vehicle while glycerine was the least effective on the basis of its MIC and not bactericidal activity. Propylene glycol was the second most effective against all microorganisms except Staphylococcus aureus.

Among all the ATCC strains of microorganisms, Staphylococcus mutans and Escherichia coli were the most susceptible to the vehicles, Enterococcus faecalis exhibited intermediate susceptibility, and Staphylococcus aureus was the most resistant to all the vehicles.

The combination of propylene glycol and PEG 400 did not show any synergistic antimicrobial activity and, in fact, its efficacy decreased against Staphylococcus mutans, Enterococcus faecalis, and Escherichia coli, in comparison to propylene glycol alone.

PEG 1000 was most effective bactericidal agent against Staphylococcus mutans and Escherichia coli among all the five vehicles and the difference was statistically significant (Fisher’s exact test: \( P = 0.003 \) and \( P = 0.004 \), respectively). For Staphylococcus aureus and Enterococcus faecalis, none of the five vehicles showed statistically significant difference in their bactericidal activity (Fisher’s exact test: \( P = 1 \) and \( P = 0.326 \), respectively).

DISCUSSION

In endodontic therapy, few cases do not respond to the conventional therapy in the pediatric and adult

<table>
<thead>
<tr>
<th>Vehicles</th>
<th>Str. mutans (%)</th>
<th>St. aureus (%)</th>
<th>En. faecalis (%)</th>
<th>Es. coli (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>50</td>
<td>100</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Glycerine</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PEG 400</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PEG 1000</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>PEG+PEG 400</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

PEG=Polyethylene glycol, PG=Propylene glycol

Figure 2: Susceptibility of Escherichia coli ATCC 25922 (a) Propylene glycol, (b) Glycerine, (c) PEG 400, (d) PEG 1000 and (e) Propylene glycol with PEG 400 assessed using Broth dilution assay

Figure 3: Minimum bactericidal concentration (MBC) of Escherichia coli ATCC 25922 (a) Propylene glycol, (b) Glycerine, (c) PEG 400, (d) PEG 1000 and (e) Propylene glycol with PEG 400 was assessed after being subcultured on BHI agar

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Endodontics. This difficulty to treat such refractory cases may be due to many reasons such as anatomic variation, presence of biofilms, and development of antibiotic resistance. To overcome the problem of the global menace of developing antibiotic resistance, the use of alternative medications and substances or a combination of pharmaceutical excipients is suggested to increase the spectrum of antimicrobial action.\textsuperscript{[14]} The most difficult to tackle is facultative anaerobes, as they are the ones that develop resistance most of the time.\textsuperscript{[11]}

The use of intracanal medicaments becomes mandatory in Endodontics as many non-vital and accessed teeth lack blood circulation. As a result of this, systemic antibiotics fail to reach the site of infection and, hence, such infections cannot be treated.\textsuperscript{[12]} Also, local drug delivery and sustained release along with better diffusion into the surrounding periapical tissues may prove to be an added advantage.\textsuperscript{[10]} The vehicles used to dispense the intracanal medicaments have a direct influence on the release, time of onset of action of the medicament, penetration of the intracanal medicaments into dental tubules, and also the dissociation of drugs. The in vitro bacterticial activity of a variety of glycols, especially monopropylene, dipropylene, and triethylene, has been investigated more extensively. The bacterticial activity of PEG 400 had been studied almost three decades ago by Vamonde\textsuperscript{[12]} and Chirfe\textsuperscript{[15]} et al. in 1982 and 1983,\textsuperscript{[17]} whereas the antimicrobial effect of PEG 1000 has been studied only recently.\textsuperscript{[15]} The antimicrobial activity of PEG 1000 might be related to the hydrophilic property of PEG. Removal of water possibly does not allow microbial growth, as a certain amount of water is essential for the multiplication and development of microbes.\textsuperscript{[15]} It is interesting to note that PEG 1000, like propylene glycol, also allowed greater dentinal penetration when combined with intracanal medicaments.\textsuperscript{[10]} After a thorough review of literature, we have not come across a single article which explores and compares the bacterticial activity of these pharmaceutical excipients.

Also, after a series of in vitro and in situ studies, the Cardiology Research Unit of the Niyasa University School of Dentistry developed the concept of 3M-RT, in which 3M stands for triple mix of antibiotics and MP stands for Macrogol, i.e., polyethylene glycol, and propylene glycol, respectively.\textsuperscript{[11,12]} This group of investigators have carried out extensive research on the most ideal antibiotics to be used for disinfection of dental tubules, but there is only one such study regarding penetration of propylene glycol as vehicle for intracanal medicament.\textsuperscript{[7]} Propylene glycol is a known antimicrobial and is an effective preservative.\textsuperscript{[7]} A recent article by Carreira et al. published in 2007 has thrown some light on the phenomenon of synergism in bacterticial activity, which was observed in Ciprofloxacin–PEG 1000 association, Metronidazole–PEG 1000 association, and Ciprofloxacin–Metronidazole–PEG 1000 combination. Also, PEG 400 produced severe plasmolysis, cell wall collapse, and finger-like extrusions in Klebsiella pneumoniae.\textsuperscript{[21]} Promising results have been shown by PEG-coated nanoparticles which were found to be most effective in killing Es. coli, St. aureus, and multi-drug resistant clinical isolates of Shigella spp. and Vibrio cholerae.\textsuperscript{[22]} Of all the vehicles being used in Endodontics, camphorated monochlophenol (CMC) is effective but tissue toxic, whereas distilled water possesses no antimicrobial activity.\textsuperscript{[7]}

In our study, all vehicles exhibited antimicrobial activity at 100% concentration. We chose broth dilution over agar well diffusion, as the diffusion of vehicles through agar would be difficult due to them being viscous. Of them, propylene glycol exhibited maximum activity against En. faecalis, which was otherwise the least susceptible to the remaining vehicles. In 1965, Oltzky reported that propylene glycol is a known antimicrobial with marked germicidal activity.\textsuperscript{[22]} Glycerine exhibited activity only at 100% concentration and, therefore, was the least effective antimicrobial. This is in accordance with the results observed by Antony et al. in 1997.\textsuperscript{[14]} Other studies demonstrated bacterticial activity at 30% and 25% concentration but at a longer incubation time, i.e., after 48 h and 7 days, which is contrary to the findings of the present study.\textsuperscript{[7]}

PEG 400, glycerine, and propylene glycol combination with PEG 400 showed similar bacterticial activity at 100% concentration only against all the selected organisms. PEG 1000 showed bacterticial activity at the lowest concentration, i.e., at 25% against Str. mutans and Es. coli, but it exhibited bacterticial activity against St. aureus and En. faecalis, the most resistant of the selected standard strains, at 100% concentration only.

Str. mutans and Es. coli were the most susceptible organisms. Susceptibility of Str. mutans and Es. coli to 25% of PEG 1000 is in accordance with the findings by Carreira et al., but the results of St. aureus and En. faecalis vary and are not in agreement, which might be due to the selection of different standard strains. The results of the antimicrobial activity of PEG 400 and glycerine are contrary to the findings of Gomez et al.,
which could be because they had used agar diffusion method to test the antimicrobial activity. The negative results may be due to their inability to diffuse through agar due to their viscous nature and not necessarily due to the absence of antimicrobial activity.[12,22] Both PEG and propylene glycol possess low toxicity and are well-recognized vehicles for drugs.[12,22,27] Also, they result in better handling properties of the resulting paste. [22] Combination of propylene glycol with PEG 400 exhibited a non-synergistic effect. PEG 400 has an inherent advantage that it does not interact with other components of the paste and exerts its antibacterial activity because it has a low water activity.[22]

CONCLUSION

The purpose of conducting this study was to test the bactericidal activity of the above-mentioned vehicles and to determine the vehicle with the maximum bactericidal activity. This will aid in enhancing the diffusion, antimicrobial activity, and release of the intracanal medicaments for a longer period of time. [13] Hence, the most effective antimicrobial vehicle has its applications in Endodontics as an intracanal medicament,[14] in Pedodontics for lesion sterilization and tissue repair,[14,13] in chronic periodontitis for local drug delivery,[14] and also in the emerging field of Regenerative Endodontics.[15] The use of vehicles with good bactericidal activity might reduce the usage of antibiotics and toxic substances like CMCP and encourage the use of innovative substances like chlorhexidine gluconate as intracanal medicaments which do not develop resistance and are more biocompatible.[12,22]

Within the limitations of this study, all the vehicles, PEG 1000 followed by propylene glycol were found to have better antimicrobial and bactericidal activities. Also, it had better handling properties of the resulting paste and diffusion through dentinal tubules.[12,22] The use of combination of propylene glycol with PEG 400 is not justified. PEG 1000 has greater bactericidal activity on S. mutans and E. coli.

Further studies should be carried out to verify and compare the diffusion of all the vehicles through dentinal tubules and against clinical isolates in vitro. Due to the synergistic action or even nullifying effect of combination of these vehicles with various intracanal medicaments, for instance, commonly used antibiotics, in vitro studies should be carried out to find the best medication and vehicle combination, followed by in vivo studies.

ACKNOWLEDGMENTS

We would like to thank Dr. Alka Kale, Dr. Shrvayogi Hugar and Dr. Sunil Jadhav for their timely help and invaluable support throughout the conduct of this study.

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Nakawade, et al: Bactericidal activity of various vehicles

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Source of Support: Nil, Conflict of Interest: None declared.
Antimicrobial Activity of Endodontic Medicaments and Vehicles using Agar Well Diffusion Method on Facultative and Obligate Anaerobes

Triveni M Nalawade, Kishore G Bhat, Suma Sogi

ABSTRACT

Aim: The aim of this study was to determine the relative antimicrobial effectiveness of these endodontic medicaments and various vehicles using an agar well diffusion assay.

Materials and Methods: Double Antibiotic Paste (DAP), modified DAP, 2% Chlorhexidine gluconate and their combination with four vehicles namely Polyethylene glycol 400 (PEG), Propylene glycol (PO), combinations of PEG with PEO and lastly Glycerine were tested using agar well diffusion assay. The minimum bactericidal concentration was noted against four standard strains of organisms ie. Streptococcus mutans ATCC (American Type Culture Collection) 25175, Staphylococcus aureus ATCC 12600, Enterococcus faecalis ATCC 35505 and Escherichia coli ATCC 25922. Successful endodontic therapy depends upon thorough disinfection of root canals. In some refractory cases, routine endodontic therapy is not sufficient, so intracanal medicaments are used for proper disinfection of canals. Issues of resistance, limited spectrum of activity and lack of antifungal properties, the hunt for the ideal intracanal medicament continues. In this regard, the vehicles used to form the pastes play a supportive role by forming the appropriate consistency for placement and may dramatically influence their chemical characteristics like their solubility and diffusion. Thus, in order to use safer and equally effective intracanal medicaments, Chlorhexidine gluconate being unveiled in this study.

Result: The difference between the four vehicles when combined with the same endodontic medicament studied above is non-significant (NS) except against Porphyromonas gingivalis. Propylene glycol is significantly effective than Glycerine when used with DAP i.e. C+M medicament combination. (p<0.029)

Conclusion: 2% chlorhexidine gluconate and modified DAP can definitely replace DAP and triple antibiotic paste as endodontic medicaments with chlorhexidine having an added advantage of bactericidal action, substantivity, biocompatibility, low toxicity, and lesser chances of developing resistance.

Keywords: Agar well diffusion, Medicaments, Minimum inhibitory concentration, Vehicles.


Source of support: Nil
Conflict of interest: None

INTRODUCTION

Dental caries is the most common chronic disease and one of the most expensive diseases to treat. As per the National Oral Health Survey and Fluoride Mapping (2002-2003), there is a very high proportion of untreated caries. Successful endodontic therapy of the teeth affected with dental caries consists of thorough disinfections of the root canals which cannot be attained by standard treatment alone. Hence, the use of endodontic medicaments for sterilization of root canals especially resistant microbes like Enterococcus faecalis has become a necessity. Although calcium hydroxide has been the most used endodontic medicaments, recently triple antibiotic paste (TAP), a combination of ciprofloxacin, metronidazole, and minocycline has been proven to be most effective. But, minocycline in TAP has been associated with discoloration and chances of affecting the developing permanent successor if used in deciduous teeth like other tetracyclines cannot be denied. Appropriate use of the existing antibiotics and also antimicrobial agents and their combinations, which help in decreasing the incidence of resistance development, should be tested and incorporated in the treatment of infectious diseases. Hence, double antibiotic paste (DAP) being an equally effective alternative, and a combination of amoxicillin clavulanate with metronidazole will be referred to as modified DAP along with 2% chlorhexidine gluconate which will be compared for their antimicrobial effectiveness. To the best of our knowledge, this is the first study to compare these three endodontic medicaments, and their combinations with various vehicles are being
Annexure – VIII – Publications

investigated to find the best combination for various uses in dentistry like intracanal medicaments, noninstrumentation endodontic therapy (NITE), and local drug delivery as used in periodontal pockets. The addition of vehicles to these intracanal medicaments not only improves the handling characteristics but also enhances diffusion through dentinal tubules, antimicrobial activity, and release of the medicaments. The aim of this study was to determine the relative antimicrobial effectiveness of these endodontic medicaments and various vehicles using an agar well diffusion assay.

MATERIALS AND METHODS

This study was carried out in Basic Science Research Centre, Belgaum. It is approved by the Institutional Review Board (Ref no. KLEU/Ethic/14-15/D-73). This study is a part of ongoing ex vivo study. The endodontic medicaments evaluated were DAP, modified DAP, 2% chlorhexidine gel and their combination with polyethylene glycol (400 PEG), propylene glycol (PG), combinations of PG with PEG, and glycerine. The antimicrobial activity was carried out against standard strains of American Type Culture Collection (ATCC) against five organisms. Streptococcus mutans was chosen as it is the most commonly isolated organism from root canals of infected teeth, whereas Staphylococcus aureus and E. faecalis were chosen as they are known to develop resistance. Porphyromonas gingivalis is also a commonly isolated Gram-negative obligate anaerobe from root canals of teeth and linked to the signs and symptoms of periapical disease and are considered to be more resistant due to outer membrane of their cell-wall structure.

Microorganisms Tested

Streptococcus mutans (ATCC 25175)
Staphylococcus aureus (ATCC 12598)
Enterococcus faecalis (ATCC 35500)
Porphyromonas gingivalis (ATCC 33277)
Escherichia coli (ATCC 25922)

Preparations of Microbial Inocula

A direct colony suspension of each test isolate was prepared and the turbidity was adjusted to 0.5 McFarland Standard, for S. mutans, S. aureus, E. faecalis, E. coli, and a 1.0 McFarland Standard for P. gingivalis.

Determination of Minimum Inhibitory Concentration of Antimicrobial Substances and their Combinations by Broth Dilution Method

Minimum inhibitory concentration (MIC) is defined as the lowest concentration where no visible turbidity is observed, i.e., bacteriostatic concentration. Brain heart infusion (BHI) broth was used for the serial dilutions. The selected microorganism was inoculated in BHI broth as per Clinical Laboratory Standard Institute (CLSI) guidelines formerly known as NCCLS, i.e., National Committee for Clinical Laboratory Standards. Also, control strains of E. coli ATCC 25922 were kept for monitoring antibacterial susceptibility testing. A known concentration of the antibacterial substances was serially diluted to two folds in broth and two controls were also included. The first contained undiluted drug which served as the positive control and other contained only inoculums which served as the negative control. Minimum inhibitory concentration was done by broth dilution method first for single drug, namely 2% chlorhexidine gluconate, ciprofloxacin, amoxicillin clavulinate, and metronidazole, and their combinations, i.e., ciprofloxacin with metronidazole (C + M) and amoxicillin clavulinate with metronidazole (A + M). For facultative anaerobes ten serial dilutions were done with incubation time of 24 hours. Whereas for P. gingivalis, 12 serial dilutions were done for both single drug and their combinations by broth dilution MIC test with incubation time of 48 hours under anaerobic conditions (Figs 1 and 2). The concentrations used were 0.5 mg/mL of ciprofloxacin, 0.5 mg/mL of amoxicillin clavulinate, and 2 mg/mL of metronidazole for single drug and 1 mg/mL concentration for both the drug combinations, i.e., C + M and A + M.

Determinations of Antimicrobial Susceptibility using Agar Well Diffusion Method

Agar well diffusion method was used to determine the antibacterial activity of endodontic medicaments and various vehicles, i.e., DAP, modified DAP and 2% CHX with PEG, PG, PEG + PG, and glycerine. A 50 μL of respective microbial inoculum was taken using a micropipette in order to provide an even lawn of cells, and loaded onto the agar plates evenly. The agar plates were inoculated with the respective microorganisms by even streaking of the swab over the entire surface of the plate three times rotating the Petri plates at 60° approximately after each applications. Finally, it was swabbed all around the periphery of the agar surface. Five wells of 7 mm size and 4 mm depth were made at an equal distance and 70 μL volume of each medicaments with respective vehicles in the ratio 1:1 (i.e., 35 μL medicaments + 35 μL vehicle) was dispensed into the four wells with the help of micropipettes. The undiluted medicaments, i.e., 70 μL of the antimicrobial substances only were dispensed into the 5th well at the center of inoculated agar plate and were considered as the positive control. The plates were then incubated at 37°C for 24 hours in an aerobic environment.
Figs 1A to D: Minimum inhibitory concentration of single antimicrobial substances, i.e.: (A) Chlorhexidine gluconate, (B) ofloxacin, (C) amoxicillin clavulanate, and (D) metronidazole by broth dilution method against P. gingivalis.

Figs 2A and B: Minimum inhibitory concentration of combination of antimicrobial substances, i.e.: (A) Ofloxacin with metronidazole, and (B) amoxicillin clavulanate with metronidazole by broth dilution method against P. gingivalis.

Figs 3A to C: Antimicrobial activity of the endodontic medicaments, i.e.: (A) 2% chlorhexidine gluconate, (B) ofloxacin with metronidazole, and (C) amoxicillin clavulanate with metronidazole along with the four vehicle combinations against P. gingivalis using agar well diffusion method.

For S. mutans, S. aureus, and E. faecalis and for 48 hours anaerobically for P. gingivalis (Figs 3A to C). The Ferri plates were observed for zone of inhibition, which were measured using a scale in millimeters. The tests were repeated three times to minimize errors.

STATISTICAL ANALYSIS
- Mean value and SD
- Kruskal-Wallis test
- Pairwise comparison using post hoc Mann-Whitney U test (p < 0.05).
RESULTS

The least MIC value of all medicaments was that of chlorhexidine and all organisms were resistant to metronidazole (Table 1). Combination of ciprofloxacin with metronidazole improved sensitivity of S. mutans, S. aureus, and P. gingivalis only while amoxicillin clavulanate with metronidazole combination improved susceptibility of S. mutans only (Table 2).

All of the selected organisms were susceptible to the antimicrobial drugs and vehicles combination except for S. mutans, which exhibited resistance to C+M and all the four vehicle combinations. There was no statistically significant difference in the same antimicrobial drug and vehicles, i.e., PEG, PG, PEG+PG, and glycerine except P. gingivalis. There existed significant difference in C+M+PG and C+M+glycerine only on P. gingivalis.

Considering drug-wise and organism-wise comparison, chlorhexidine was significantly more effective than C+M and A+M against S. mutans and S. aureus. C+M was the most effective on E. faecalis followed by chlorhexidine; whereas A+M was most effective against P. gingivalis, thus justifying the combination for obligate anaerobes.

DISCUSSION

The sterilization and disinfection of the root canal systems consists of reduction in microbes to enable local response and ensure healing of damaged tissues. Studies of the microbial diversity of the root canal infections demonstrate that relative proportions of anaerobes increase with time and that facultative anaerobes increase when root canals remain infected for longer periods. The most predominantly isolated organisms is S. mutans followed by facultative microbes, such as E. faecalis and S. aureus, which constitute the most resistant species. E. faecalis is one of the possible causes of root canal failure. Recently, E. faecalis has been predominantly found in primary endodontic infections too. Also, Gram-negative anaerobes, e.g. black-pigmented species have been associated with signs and symptoms of periodontal disease and exhibit resistance due to the outer membranes of their cell wall.

The advantage of local application of an antibiotic is that it permits the use of very large doses, hence being bactericidal, thus overcoming resistance without risk of systemic toxicity to the subject as the overall dose is small. Hence, depending on the MIC of combination of drugs by broth dilution method, higher dosage of 100 μg/mL was chosen. Also, previous studies have shown the dose of 100 μg/mL to be bactericidal and no bacteria were recovered from the sample. However, concentrations of 1 and 10 μg/mL allowed some microbes to persist, i.e., these concentrations were only bacteriostatic.

Chlorhexidine has been used extensively in endodontics for irrigation and as an intracanal medicament and is considered as the gold standard. It is a cationic biguanide and an antiseptic. It has substantive properties and even at higher concentrations has very low toxicity. At low concentration, it is bacteriostatic, whereas at high concentration, it is bactericidal. It shows antimicrobial activity from concentrations as low as 0.1% and shows bactericidal activity at 2% and is biocompatible.

The MIC of chlorhexidine started from 0.019% against P. gingivalis to 0.078% against other facultative anaerobes with the highest concentration against E. faecalis, i.e., 0.155%. Similar results were observed by Misery et al., i.e., <0.0625% of chlorhexidine against facultative anaerobes but in case of E. faecalis, our study shows higher values though the standard strain of E. faecalis is the same and the MIC also has been determined in both the studies by broth dilution technique. Strict anaerobes, e.g., P. gingivalis as in our study were most susceptible to
chlorhexidine without the vehicles, which is contrary to the findings of Filho et al.⁶ Chlorhexidine when studied with vehicles by agar well diffusion method showed lesser zones of inhibition of P. gingivalis as compared to other microbes due to being more effective against Gram-positive organisms than Gram-negative.⁷

The MIC of ciprofloxacin alone and decrease in MIC when combination of C+M are in unison with the results stated by Carreira et al.⁸ except for S. aureus might be due to the use of different ATCC strains. The zones of inhibition of ciprofloxacin against clinical isolates of S. aureus and E. faecalis are greater at drug concentration of 5 µg/ml⁹ as compared to 100 µg/ml in our study against standard strains of same microorganisms. It is interesting to note that standard strains of S. mutans exhibited resistance C+M along with the four vehicles when tested by agar well diffusion method which is in accordance to study by Jain et al.¹⁰ Double antisepctic paste has been shown to be equally effective as TAP¹¹ and it was observed that MIC of C+M against all organisms except E. faecalis was lower as compared to ciprofloxacin alone. Another study by Choudhary et al.¹² shows amoxicillin and ciprofloxacin to be highly effective in terms of zones of inhibition, i.e., 31 and 30 respectively, whereas tetracyclines to be moderately effective against clinical isolates of S. mutans.¹³

As per the principles of antibiotic therapy, the use of narrow spectra but bactericidal drugs can prevent the development of drug resistance. Combination of two bactericidal drugs can overcome selective pressure and avoid development of resistance in microorganisms. Beta-lactamase production is the most common reason for resistance in Enterobacteriaceae, so clavulanic acid which is a beta-lactamase inhibitor can be used.¹⁴ Hence, the selection of amoxicillin clavulanate, instead of ciprofloxacin has also been found to be 100% effective against endodontic bacteria.¹⁵ Therefore, modified DAP with amoxicillin clavulanate was studied for the first time for lesion sterilization and tissue repair or noninstrumentation endodontic technique.

The MIC of amoxicillin clavulanate alone is in accordance with the guidelines by Indian Council Medical Research, except for P. gingivalis and E. coli.¹⁶ Also, the MIC combination of amoxicillin clavulanate with metronidazole improved or stayed consistent for all organisms except S. aureus. Additionally, the zones of inhibition of A+M with the four different vehicles were the lowest, i.e., against P. gingivalis which is similar to findings by Coscon et al.¹⁷

The zones of inhibition were greater for P. gingivalis as obligate anaerobes are rather easily eradicated and metronidazole targets anaerobes in particular.¹⁸ Additionally, 2% CHX was unanimously effective against all pathogens with additional advance of no resistance development being a cation.¹⁹

The use of vehicles like PG not only enhances the penetration of the drugs into their dentinal tubules as observed by Cruz et al.²⁰ but also can even make microbes having drug resistance; sensitive when used along with vehicles like PEG, e.g., metronidazole when used with PEG as in study by Carreira.²¹ The use of vehicles as carriers for intracanal medicaments also improve handling properties of the resulting paste and enhance their release too. All the above selected vehicles do possess antimicrobial activity.²²

No significant difference in vehicles when mixed with endodontic medicaments except for PG in comparison with glycine when used along with C+M combination of drugs against P. gingivalis (Table 3) as these vehicles

<table>
<thead>
<tr>
<th>Antimicrobial drug</th>
<th>Vehilce</th>
<th>S. mutans(mm)</th>
<th>S. aureus(mm)</th>
<th>E. faecalis(mm)</th>
<th>P. gingivalis(mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorhexidine PEG</td>
<td>31.00(1.00)</td>
<td>38.00(1.00)</td>
<td>31.67(1.52)</td>
<td>26.00(1.00)</td>
<td></td>
</tr>
<tr>
<td>PEG</td>
<td>32.00(1.00)</td>
<td>37.67(1.15)</td>
<td>26.33(0.57)</td>
<td>27.00(1.00)</td>
<td></td>
</tr>
<tr>
<td>PG + PEG</td>
<td>31.23(0.57)</td>
<td>37.57(0.57)</td>
<td>28.00(1.00)</td>
<td>27.57(1.52)</td>
<td></td>
</tr>
<tr>
<td>Glycerine</td>
<td>31.23(0.57)</td>
<td>37.57(0.57)</td>
<td>28.00(1.00)</td>
<td>27.57(1.52)</td>
<td></td>
</tr>
<tr>
<td>H-Value*</td>
<td>1.90</td>
<td>7.30</td>
<td>0.74</td>
<td>3.37</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.50(NS)</td>
<td>0.033(NS)</td>
<td>0.079(NS)</td>
<td>0.337(NS)</td>
<td></td>
</tr>
<tr>
<td>C+M</td>
<td>26.00(1.00)</td>
<td>34.00(1.00)</td>
<td>37.00(1.00)</td>
<td>37.00(1.00)</td>
<td></td>
</tr>
<tr>
<td>PEG</td>
<td>24.00(1.00)</td>
<td>30.00(1.00)</td>
<td>38.33(0.57)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>24.00(1.00)</td>
<td>32.00(1.00)</td>
<td>39.00(1.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG + PEG</td>
<td>23.67(0.57)</td>
<td>32.67(0.57)</td>
<td>32.57(1.52)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerine</td>
<td>23.67(0.57)</td>
<td>32.67(0.57)</td>
<td>32.57(1.52)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-Value*</td>
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<td>3.041</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.385(NS)</td>
<td>0.385(NS)</td>
<td>0.385(NS)</td>
<td>0.298*</td>
<td></td>
</tr>
</tbody>
</table>

*Kruskal Wallis test, *p<0.05 non significant (NS), *p<0.05 significant, *pairwise comparison using post hoc Mann Whitney U test statistically significant (p<0.05). All other pairwise comparisons are non significant (NS)
might not be having a synergistic effect when used with the three intracanal medicaments, i.e., 2% chlorhexidine gluconate, C-M and A-M.

The agar well diffusion method or well plate method or the agar diffusion method was used in this study as it is the most commonly used method of antimicrobial activity determination especially of newer substances like plant extracts, new drug formulations, etc. This technique is a well-accepted way of comparing the antibacterial effect of different dental materials, medicaments, etc. Ager well diffusion was used for the combination of antimicrobial drugs with vehicles as though E-test being the latest method, it is not feasible for combination of drugs.

CONCLUSION

The many hurdles in using antibiotics as intracanal medicaments are: Issues of resistance, limited spectrum of activity, lack of antifungal properties, and the hunt for the ideal intracanal medicament continues. In this regard, the vehicles used to form the pastes play a supportive role by forming the appropriate consistency for placement and may dramatically influence their chemical characteristics like their solubility and diffusion. Though difference between the four vehicles studied above is NS except P. gingivalis, PC5 or PEG36 can be used to improve diffusion and slow release of medicaments for longer period of time. The inhibitory effect of PEG is definitely an advantage against Gram-negative species when used as a base for the formulation of endodontic medicaments.

Hence, 2% chlorhexidine gluconate and modified DAP can definitely replace DAP and TAP as endodontic medicaments with chlorhexidine having an added advantage of bactericidal action, substantivity, biocompatibility, low toxicity, and lesser chances of developing resistance. Therefore chlorhexidine can be used for facultatively anaerobic bacterial species, like S. mutans, and also for species known to develop resistance like E. faecalis and S. aureus. Modified DAP was the most effective against obligate anaerobes P. gingivalis, but it was also sensitive to 2% chlorhexidine gluconate.

Furthermore, ex vivo studies should be carried out as agar diffusion may be influenced by physical-chemical properties of the medicaments, nature of agar, composition, porosity, pH, and thickness of agar media. Ex vivo studies will give us an appropriate understanding of the diffusion through dentinal tubules of these newer combinations of endodontic medicaments and vehicles.

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Annexure – VIII – Publications

PR08-1.28

In-vitro antibacterial activity of innovative endodontic medicaments and different vehicle combinations

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Background: Dental caries is the most common chronic disease and one of the most expensive diseases to treat. The National Oral Health Survey and Fluoride Mapping 2002–2003 in India, revealed a high proportion of untreated caries. The reasons being the cost of treatment, myths about the primary dentition that they shed off and do not require treatment. Additional influencing factors were that dental treatment for children is multi-visit and causes loss of pay on part of the parents to avail treatment at educational institutions. Hence, Lesion Sterilization and Tissue Repair with the combination of antibacterial agents for disinfection of pulpal and periapical lesions of primary teeth might be a simple and economical treatment with more patient acceptance.

Aim: The aim of this study was to evaluate antibacterial activity of innovative endodontic medicaments and different vehicle combinations using well-plate method.

Design: In-vitro study on Streptococcus mutans (ATCC 25175), Staphylococcus aureus (ATCC 12598) and Enterococcus faecalis (ATCC 35550).

Results: 2% Chlorhexidine gluconate was consistently effective of all endodontic medicaments especially in combination with propylene glycol (P < 0.05) amongst all vehicles followed by a mixture of propylene glycol and polyethylene glycol.

Conclusions: 2% Chlorhexidine gluconate in combination with propylene glycol could be also used as an endodontic medicament instead of the routinely used antibiotic pastes in Lesion Sterilization and Tissue Repair. Chlorhexidine gluconate is a safer alternative to the routinely used Triple Antibiotic paste and Double Antibiotic Paste due to its inherent advantage of not developing resistance in micro-organisms and low toxicity.
ANNEXURE – IX – CERTIFICATE AS GUEST SPEAKER

[Image of the certificate]

Certificate of Appreciation

To Dr. Triveni Mohan Nalawade

In honour of outstanding and informative lecture as a Guest Speaker

On 20th June, 2015

Sri Venkata Sai Institute of Dental Sciences, Mahabubnagar, Telangana
ANNEXURE – X – CONFERENCES ATTENDED

Certificate of Attendance

This is to certify that

[Signature]

attended the

25th Congress of the International Association of Paediatric Dentistry, Glasgow, UK from 1st – 4th July 2015

and presented

In-vitro antibacterial activity of innovative endodontic medicaments and different vehicle combinations

Signed:

[Signature]

Richard Welbury
Chair, IAPD 2015 Organising Committee
This Certificate of Appreciation is awarded to

Dr Triveni Mohan

Has actively participated as a speaker in 18th Oman International Dental Conference held on 31st March to 1st April 2016 at Grand Hyatt Hotel-Muscat, Oman.

It is certified that the above participant is entitled to claim 5 Credit points under category 1.

CPD registration no. OMSR/CPD/7456/10.36

Dr. Salehudeen Al Balooshi
Head of Scientific Committee

Dr. Hussain Al-Lawati
Chairman of Oman Dental Society
Chairman of the organizing committee, 18th Oman International Dental Conference
ANTIBACTERIAL ACTIVITY OF INNOVATIVE ENDODONTIC MEDICAMENTS AND DIFFERENT VEHICLE COMBINATIONS:
AN IN-VITRO STUDY

Date: Friday 1 April 2016, Time: 16:40 – 16:55
Venue: Al Afrah A Ballroom

Aim: The aim of this study was to evaluate antibacterial activity of innovative endodontic medicaments and different vehicle combinations using an agar well diffusion assay. Design: In-vitro study on Streptococcus mutans (ATCC 25175), Staphylococcus aureus (ATCC 12598), Enterococcus facealis (ATCC 35550) and Eschericia coli (ATCC 25922).

Results: All the combinations of endodontic medicaments were effective but there was no significant difference between the medicaments. Amongst all vehicles propylene glycol and polyethylene glycol were significantly better as carriers for endodontic medicaments as compared to their combination and glycerine (p<0.05).

Conclusions: Antibiotics could be attempted to be replaced by 2% Chlorhexidine gluconate in combination with either propylene glycol or polyethylene glycol could be also used as an endodontic medicament in Regenerative endodontics for revascularization as also in non-instrumentation endodontic technique. Antiseptics like Chlorhexidine gluconate is a safer alternative to the routinely used Triple Antibiotic paste and Double Antibiotic Paste due to its inherent advantage of not developing resistance in micro-organisms and low toxicity.

LEARNING OBJECTIVES:
1) Provide an overview of recent endodontic medicaments and the vehicles used as carriers.
2) Review of their use in Regenerative Endodontics for revascularization of young permanent teeth.
3) Use of alternatives to antibiotics to overcome problems of antibiotic resistance.