ASSESSMENT OF NITROFURANTOIN AS AN EXPERIMENTAL INTRACANAL MEDICAMENT IN ENDODONTICS

A THESIS SUBMITTED TO THE COUNCIL OF COLLEGE OF DENTISTRY, UNIVERSITY OF SULAIMANI IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN CONSERVATIVE DENTISTRY

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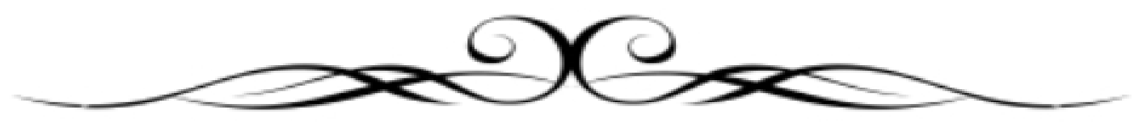

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Dedication

This PhD research thesis is dedicated to all members of my beloved family, especially to: my Adorable (Father & Mother), my Lovely Husband (Dr. Ali Kamal), my Wonderful Sons (Rozhyar & Karzheen) and to my Beautiful Daughters (Yara & Lareen)
For their supports, prayers, encouragement, advices and endless love that have sustained me throughout my life.

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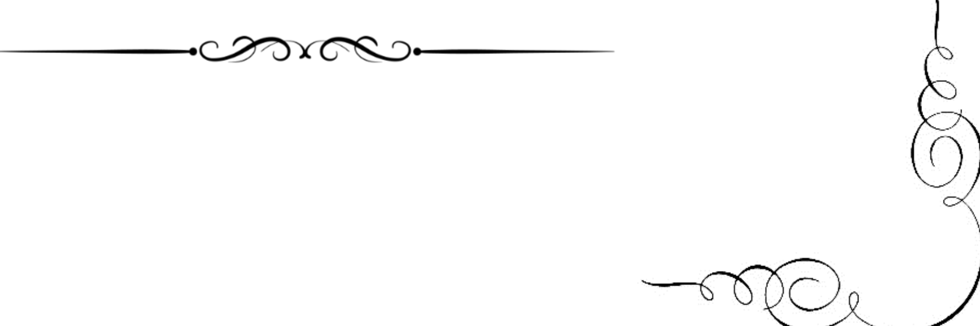

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ABSTRACT:

Background and objectives: Multiple antibiotic agents have been combined to be used as intracanal medicament, such as modified triple antibiotic paste (MTAP), to eliminate *Enterococcus faecalis* (*E. faecalis*) which is the most frequently identified bacteria in failed root canal treatment. However, due to MTAP drawbacks, there is a search for new agents. Thus, this study aimed to evaluate a single antibacterial agent, nitrofurantoin (Nit), as an experimental intracanal medicament paste against *E. faecalis*, its biocompatibility in rats' subcutaneous tissue, and its effect on the microhardness of radicular dentin, and compare the results with those of MTAP.

Materials and methods: Three clinical isolates of *Enterococcus faecalis* (S1, S2, and S3) were used. The antibacterial efficacy of nitrofurantoin and modified triple antibiotic mixture (ciprofloxacin, clindamycin, and metronidazole) against the three isolates of *E. faecalis* was assessed using serial dilution method to determine The MIC (minimal inhibitory concentration) and the MBC (minimal bactericidal concentration) of those antibiotics, in addition using agar diffusion method to measure the zone of inhibition. For evaluation of the antibacterial efficacy of nitrofurantoin paste in an *ex-vivo* assay, a total of 198 single-rooted human teeth were collected and divided randomly into three main groups; Group N (Nit) (n=90), group M (MTAP) (n=90), and group W (distilled water) (n=18). The main groups were subdivided into three subgroups according to the isolates of *E. faecalis*. Then each subgroup of N and M was subdivided into five groups (n=6) according to the concentration of Nit or MTAP (6.25, 12.5, 25, 50, and 100 mg/mL). For the biocompatibility assessment, 24 Wistar rats were divided into four experimental periods groups. Sterile polyethylene tubes were filled with 12.5mg/mL Nit paste as tested group 1 (TG1), 25 mg/mL Nit paste as

tested group 2 (TG2), and 25 mg/mL MTAP as a positive control group (PC) and implanted subcutaneously. Empty tubes were implanted as a negative control group (NC). After the experimental periods, animals were euthanized and the implantation sites were excised, and the specimens were subjected to histochemical preparation. For the microhardness evaluation, 18 human canine roots were instrumented and divided into three groups; 25mg/mL Nit paste, 25mg/mL MTAP and negative control (distilled water). The roots were transversally sectioned into three thirds (coronal, middle, and apical). The effect of each paste on the microhardness of radicular dentin was measured using a Vickers Microhardness tester, before and after treatment.

Results: The MBC result of Nit and MTAP was found to be similar to the MIC for all the isolates. The agar diffusion method showed that, the diameter of the zone of inhibition was directly proportional to the concentration of both medicaments, and it was largest in S1, and smallest in S3. In ex-*vivo* assay the result showed that nitrofurantoin paste could eradicate the three isolates of *E. faecalis* (S1, S2, and S3) completely with concentrations of (6.25, 12.5, and 25mg/mL), respectively. While MTAP showed complete eradication of the three isolates only at 25mg/mL. The result of the biocompatibility evaluation revealed that the intensity of inflammatory response was more in PC than the two testing groups; (TG1 and TG2) in all period intervals. In testing groups, the intensity of inflammatory response was more in TG2 than TG1 in all period intervals. The intensity of inflammatory response in NC was less than the other groups. Regarding the microhardness evaluation, 25mg/mL MTAP showed a significantly more percentage of reductions in microhardness of radicular dentin (9.18 %) than 25mg/mL Nit paste (1.88 %), when compared with control group.

Conclusion: At the concentration of (25 mg/mL), nitrofurantoin paste could eradicate *E. faecalis* completely, when it is used as an intracanal medicament, additionally, it is biocompatible and minimizes the reduction in microhardness of the radicular dentine compared with the 25mg/mL MTAP

Keywords: *Enterococcus faecalis*, Intracanal medicament, Microhardness, Modified triple antibiotic paste, Nitrofurantoin.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
AS	Aggregation substance
AST	Antimicrobial susceptibility testing
BHI	Brain heart infusion
C. albicans	*Candida albicans*
CFU	Colony Forming Unit
CH	Calcium hydroxide
CHX	Chlorohexidine
CLSI	Clinical and Laboratory Standard Institute
DAP	Double antibiotic paste
DNA	Deoxyribonucleic acid
DW	Distilled Water
EDTA	Ethyl Dimethyl Tetra Acetic acid
ESP	Enterococcal surface protein
FDA	Food and drug administration
KHN	Knoop Hardness Number
MBC	Minimal bacteriocidal concentration

MC	Methylcellulose
MIC	Minimal inhibitory concentration
MTAP	Modified Triple Antibiotic Paste
Ncm	Newton centimeter
Nit	Nitrofurantoin
PCR	Polymerase chain reaction
pH	Potential Hydrogen
PSI	Pound per square inch
RNA	Ribonucleic acid
SD	Standard Deviation
Spp	Species
TAP	Triple antibiotic paste
HV	Microhardness value
UTIs	Urinary tract infections

INTRODUCTION:

Enterococcus faecalis (*E. faecalis*), facultative bacteria, is the most predominant and most resistant microorganism leading to persistent periradicular lesions and eventually endodontic failure (Sedgley *et al*. 2006; Rôças *et al*. 2008; Wang *et al*. 2012a). It is found in root canal failures in nearly (24–70%) by culturing methods (Hancock *et al*. 2001; Adib *et al*. 2004; Gomes *et al*. 2004), and in (67–77%) by molecular methods (Rôças *et al*. 2004b; Siqueira and Rôças 2004; Sedgley *et al*. 2006; Gomes *et al*. 2008). In other studies, it was retrieved as a major component, about 90% (Dahlen *et al*. 2000; Fouad *et al*. 2005). This microorganism owns many special properties that enable it to survive in root canal and cause re-infection such as the ability to tolerate periods of starvation, deeply invade dentinal tubules (Donlan *et al*. 2002), antimicrobial resistance, and the ability to adapt to changing environment (Stuart *et al*. 2006).

Enterococcus faecalis can survive inside the complex anatomy of the root canal system even after chemo-mechanical preparation of root canal (Shuping et al. 2000; Demiryurek *et al*. 2014). Therefore, the intracanal medicaments represent an ideal reinforcement step to achieve the complete disinfection of the root canal system (Kumar *et al*. 2019).

Local application of antibiotics, in form of intracanal medicaments, has been an option for years in endodontics to combat endodontic infections (Abbott *et al*. 1990). It represents a more successful route than systemic antibiotics to prevent the risks of adverse side effects of antibiotics (Mohammadi and Abbott 2009). Also it gives the chance to target bacteria in each fine locus of the root canal system, which cannot be reached by conventional root canal treatment protocols such as instrumentation and irrigation (Segura-Egea *et al*. 2017). Moreover, it can be used in higher drug concentrations locally (Bansal *et al*. 2014). However, this technique may

face a problem, the emergence of bacterial resistance (Slots 2002; Huang 2008).

Triple antibiotic paste (TAP), which is a combination of three antibiotics, namely: metronidazole, ciprofloxacin, and minocycline, has been used as an intracanal medicament owing to its high antimicrobial effects (Parhizkar *et al*. 2018). There is a controversy between the studies supporting its efficacy to eradicate *E. faecalis* in the root canal system completely (Mozayeni *et al*. 2014; Ravi 2017; Ghabraei *et al*. 2018; Sabarathinam *et al*. 2018). This may be due to emerging bacterial resistance (Slots 2002; Huang 2008). Another drawback of TAP is the crown discoloration due to its Minocycline (Kirchhoff *et al*. 2015; Jagdale *et al*. 2018). Therefore, there has been a modification of TAP called modified triple antibiotic paste (MTAP) (Karczewski *et al*. 2018), by replacing minocycline with clindamycin. MTAP was shown to be as effective as TAP in reducing *E. faecalis* in the root canal system (Algarni *et al*. 2015). Several studies reported that TAP and MTAP showed significant reductions in microhardness of radicular dentin due to their acidic pH resulting in demineralization of dentine surface in addition to changes in the chemical structure of dentine (Eliades 1994; Prather *et al*. 2014; Yassen *et al*. 2015; Nerness *et al*. 2016).

Due to those mentioned drawbacks of TAP and its modification, there was a need for a new medicament that has equivalently potent against *E. faecalis*, less possibility of developing bacterial resistance, less negatively effective on the microhardness of the radicular dentin, preferably being single drug rather than multi-drug, so needs less time and efforts to prepare, additionally cost-effective.

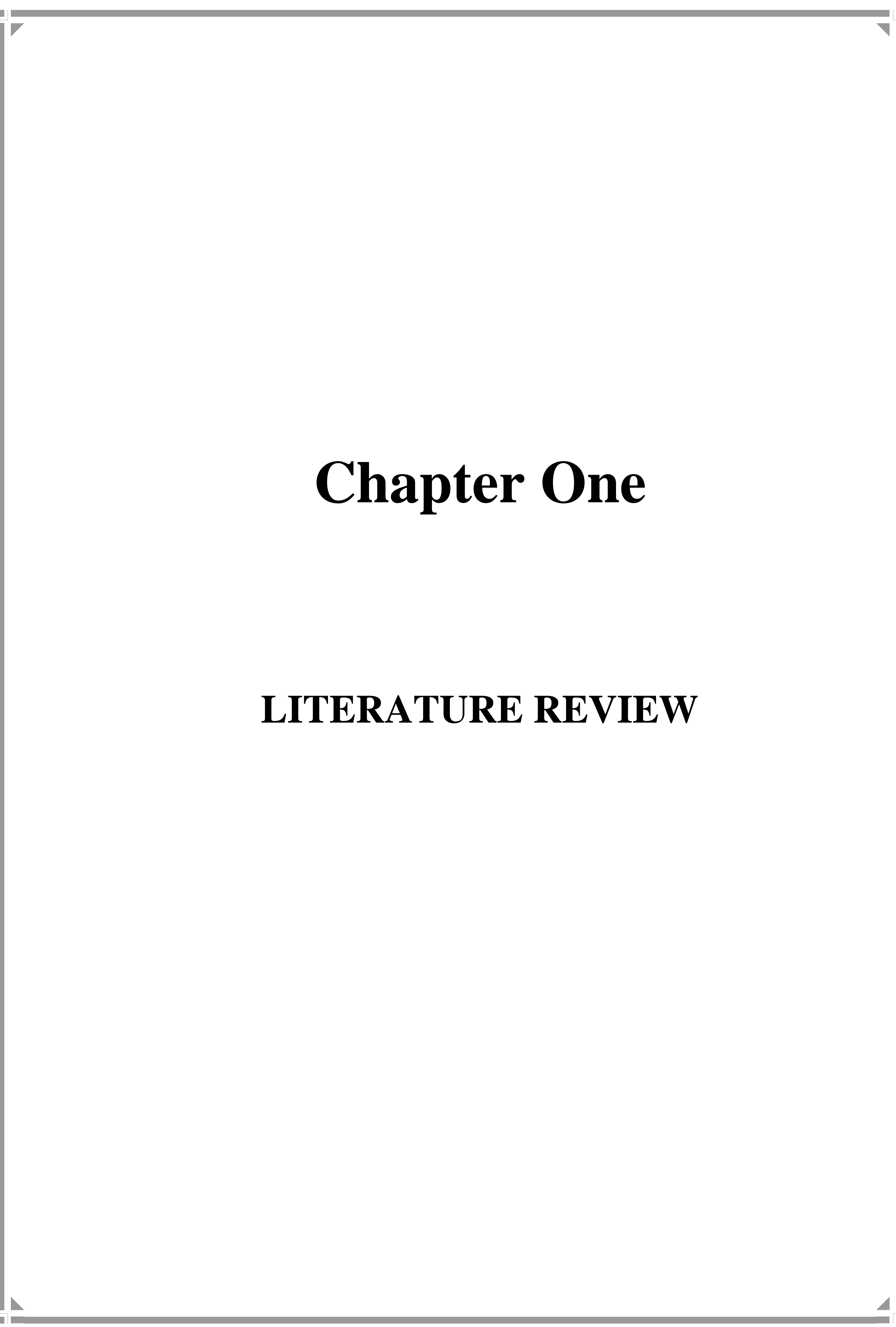
Nitrofurantoin (Nit) is a synthetic nitrofuran compound (Cunha 1989). It is effective against most gram-positive and gram-negative organisms (Qiao *et al*, 2013). It is a well-known antibacterial agent widely used as an oral

antibiotic treatment for urinary tract infections (UTIs) (Asadi Karam *et al*. 2019). Furthermore, it is the drug of choice for the treatment of infections caused by multidrug-resistant pathogens (Munoz-Davila 2014; Shakti and Veeraraghavan 2015; Gardiner *et al*. 2019). Several studies confirmed that nitrofurantoin is highly effective against *E. faecalis* (Abdulla and Abdulla 2006; Rahbar *et al*. 2007; Toner *et al*. 2016). No previous studies have been done to explore the effect of nitrofurantoin as a new intracanal medicament within the root canal system against *E. faecalis* in endodontics. Therefore, this study was designed to assess the antimicrobial efficacy of nitrofurantoin paste as an intracanal medicament against *E. faecalis*, and to evaluate the effect of nitrofurantoin paste on the microhardness of the radicular dentin, and compared it with MTAP. In addition, this study was also carried out to evaluate the biocompatibility of nitrofurantoin paste in subcutaneous connective tissue of rats, as any new formulations in endodontics have to be tested in vivo before applying to human beings (Shahi *et al*. 2019).

OBJECTIVES OF THE STUDY:

1. To measure the antimicrobial efficacy of nitrofurantoin as an experimental intra canal medicament against three isolates of *Enterococcus faecalis* bacteria in comparison with modified triple antibiotic paste (MTAP), using three different methods:
    a. Serial dilution method to evaluate the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC).
    b. Kirby-Bauer test to measure the zone of inhibition.
    c. Counting viable bacteria in the root canal of extracted teeth. (Ex-*vivo* study)

2. To measure the pH of nitrofurantoin and compare it with modified triple antibiotic mixture.
3. To evaluate the biocompatibility of nitrofurantoin paste in rats' subcutaneous tissue.
4. To investigate the effect of nitrofurantoin paste on the microhardness of radicular dentin in comparison with modified triple antibiotic paste (MTAP).



Chapter One

LITERATURE REVIEW

Literature review

1.1 Endodontic treatment:

Endodontic treatment or endodontic therapy is a series of steps for treatment of the infected pulp of a tooth aiming at eradication of infection and prevention of the decontaminated tooth from futuristic microbial infestation (Kumar *et al*. 2019). Successful endodontic treatment involves: a correct diagnosis and establishing an individualized treatment, orientation of the morphology and anatomy of tooth and appropriate instrumentation, disinfection, and obturation of the whole root canal system (Estrela *et al*. 2014; Torabinejad *et al*. 2015). So, the biological purpose of endodontic treatment is to eliminate pathogenic microorganisms from the root canal system with shaping of the canal system appropriately and obturate it with a suitable material (Vasudeva *et al*. 2017).

The major factor associated with endodontic failure is the persistence of microbial infection in the root canal system and periradicular area (Rôças *et al*. 2008; Wang *et al*. 2012a). Chemomechanical preparation is considered the first step to eliminate the pathogenic intracanal bacterial species (Hulsmann *et al*. 2005). Since chemomechanical preparation minimizes the bacterial count in the canal, none of the available techniques can completely eradicate the bacteria from the root canal system. Therefore, intracanal medicaments are used to eliminate and/or reduce the number of bacteria from the canal (Carbajal and Arrieta 2016; Gokturk *et al*. 2016).

The selection of the intracanal medicament relies on the accurate diagnosis of the causative microorganisms, in addition to knowing their mechanisms of growth and survival. The use of an antimicrobial agent is

justified by the fact that the condition is mostly caused by persistence of bacteria within the root canal (Murvindran and Raj 2014).

Nowadays, research is directed towards investigating the most resistant organisms and the procedures to eradicate them (Narayanan and Vaishnavi 2010).

1.1.1 Failure of endodontic treatment:

The failure of endodontic treatment can be diagnosed depending on a group of clinical signs and symptoms in addition to the radiographic appearance of treated root canal (Iqbal 2016). Thus, endodontic failure means a recurrence of clinical symptoms and the presence of a periapical radiolucency (Tabassum and Khan 2016).

Microbial infection is blamed as the main cause for endodontic failure, and associated by clinical signs and symptoms such as pain, periapical lesion, swelling, tenderness to percussion, and sinus tract. These symptoms are the result of the occurrence of bacterial irritants leading to an inflammatory response of the periapical tissues (George and Ivančaková 2007; Vineet *et al*. 2016).

Failure of endodontic therapy when there is insufficient instrumentation, irrigation and/or over, under obturation. Olcay *et al*. (2018) and Misgar *et al*. (2018) showed that the outcome of root canal treatment is significantly affected by the quality of obturation and the presence and quality of coronal restoration.

Primary endodontic treatment has a high success rate, nevertheless, failures still found because of inadequate knowledge and skill for performing endodontic treatment or from not abidance of evidence based guidelines and principles of endodontics (Misgar *et al*. 2018).

Endodontic retreatment has less success rates compared to the primary endodontic treatment. The success rate of endodontic retreatment reaches about 66%, which is less than that of primary endodontic treatments. This is due to many possible causes like technical difficulties leading to iatrogenic factors and the inability to eradicate microorganisms which are resistant to biomechanical preparation (Santos *et al*. 2019).

1.1.2 Factors inducing failure of endodontic treatment:

Failure of primary endodontic treatment is usually caused by several factors which include:

- Bacterial persistence (intraradicular and extraradicular).
- Residual necrotic pulp tissue.
- Inadequate or overextensions of root filling materials; obturation was considered unsatisfactory if the end of the obturation filling was more than 2 mm short of the apex or had extruded beyond the apex; and space was visible laterally along the obturated canal or voids were present within the filling mass.
- Inadequate access opening ending in missed canals or unfilled canals (both major and accessory).
- Improper coronal seal; microleakage of temporary or definitive coronal restoration. Unsatisfactory restorations characterized by any secondary carious lesions, marginal defects that the explorer could penetrate, or any fracture or loss of the restoration.
- Complications due to instrumentation such as ledges, zipping, perforations and root fractures, or broken instruments.
- Anatomy complexity of the root canal system; might cause failure even if root canal treatment was adequately performed, as non-instrumented

areas of root canals may keep bacteria and necrotic tissue even though not visible on radiograph (Wang *et al*. 2012b; Iqbal 2016; Tabassum and Khan 2016; Misgar *et al*. 2018).

1.2 Endodontic infection:

Endodontic infection is the infection of the root canal system and is the main causative etiology of apical periodontitis (Siqueira and Rocas 2008).

Endodontic infections are different from other oral infections as they take place in a closed environment, surrounded by hard tissues. Endodontic infections occur because the root canal system gets exposed to the oral environment accompanied by a decline in the body's immune system (Singh 2016). Bacteria are the most common microorganisms causing endodontic infections, which to a less extent might be caused by fungi, archaea, and viruses (Siqueira and Rocas 2009).

Antony van Leewenhoek was the first to observe oral flora (1632-1723). He described the "animalcules" which he observed through his microscope and were isolated from dental plaque and exposed pulp cavity (Kuramitsu *et al*. 2007). In 1894, Miller WD published his findings on the bacteriological investigation of root canal infection (George and Ivancakova 2007).

Endodontic bacteria are found in all parts of the root canal system at varying depths of up to 300 µm within the dentinal tubules, from the canal lumen. There is a regional variation in bacterial invasion of the dentine; cervical tubules are invaded the most, then the midroot tubules, and finally, the apical tubules being the least to be invaded (Coldero *et al*. 2002; Demiryurek *et al*. 2014). The bacteria in the periradicular tissues can invade and reside in the cementum around the periapex (Vijayaraghavan *et al*. 2012).

1.2.1 Pathway of endodontic infection:

The most common cause of pulpal inflammation (pulpitis) is bacteria or their products which may get access into the pulp through a deep caries lesion or a leaking filling (Haapasalo *et al*. 2005; Rechenberg *et al*. 2016).

The microorganisms can enter the pulp through: dentinal tubules, lateral and accessory canals, open cavity, faulty restoration, apical foramen, periodontal membrane, or through blood stream (Sunitha *et al*. 2008; Fouad 2017).

The most common route of contamination is dental caries, which can lead to progressive inflammatory responses in the pulp tissue, resulting in pulp necrosis in case that adequate therapeutic measures are not performed. For an infection to occur, the microorganisms should withstand physical and biological barriers and survive within the pulp space (Dudeja *et al*. 2015; Gomes and Herrera 2018).

1.2.2 Classification of endodontic infection:

Endodontic infection can be classified according to the anatomical location; intraradicular and extraradicular. Intraradicular infections are subdivided into; primary, secondary/persistent infections, depending on the onset of appearance of pathogenic microorganisms within the root canal. The composition of the microbiota may vary depending on the different types of infection (Patel and Barnes 2013; Torabinejad *et al*. 2015).

1.2.2.1 Intraradicular infection:

Primary and secondary/persistent endodontic infections are mostly located intraradicularly, and if left untreated or inadequately treated, they may extend to form extraradicular infections (Gomes and Herrera 2018).

1.2.2.1.1 Primary intraradicular infection:

Primary intraradicular infection is defined as the infection caused by initial invasion and colonization of the necrotic pulp tissue by microorganisms (Bouillaguet *et al*. 2018). In other terms, primary intraradicular infection is an untreated canal where the pulp tissue is colonized by microorganisms. Progression of pulpal infection depends on many factors such as number of bacteria, caries, trauma, and iatrogenic variables (Gajan *et al*. 2009).

This infection can be caused by carious or traumatic exposure of coronal pulp or any other breach in the hard tissue integrity of the tooth structure. Microorganisms, can cause acute or chronic infections, depending on their virulence and host tissue defense mechanism (Singh 2016).

Molecular and cultural studies have shown that the microorganisms found in primary endodontic infections are mainly anaerobic bacteria, furthermore, an infected root canal may contain from 10 to 30 bacterial species, (Munson *et al*. 2002; Siqueira and Rocas 2005; Siqueira and Rocas 2009) and those microorganisms colonizing the apical region may have a predominant role in the etiology of apical periodontitis (Gomes and Herrera 2018).

Major bacterial groups and species that are responsible for primary intraradicular infection are summarized in (Table 1.1) (Siqueira and Rocas 2009; Narayanan and Vaishnavi 2010; Singh 2016).

Other pathogens found in primary endodontic infections are fungi (particularly, *Candida spp*. (e.g.,) *Candida albicans*), and most recently archaea and viruses and as yet uncultivable bacteria have been found in association with endodontic infections (Patel 2015a; Teles *et al*. 2013; Singh 2016).

Table 1.1 Bacteria causing primary intraradicular infections. (Siqueira and Rocas 2009; Narayanan and Vaishnavi 2010; Singh 2016)

MAIN GROUP OF BACTERIA SPECIES	
Black pigmented Bacteria (a) saccharolytic species – *Prevotella* (b) asaccharolytic species -*Porphyromonas.*	*Prevotella* species detected in endodontic infections include • *Prevotella intermedia* • *Prevotella nigrescens* • *Prevotella tannerae* • *Prevotella multissacharivorax* • *Prevotella baroniae* • *Prevotella denticola* *Porphyromonas* species detected in endodontic infections include • *Porphyromonas endodontalis* • *Porphyromonas gingivalis*
Tannerella forsythia : (previously called *Bacteroides forsythus* or *Tannerella forsythenis)* Was the first periodontal pathogen to be detected in endodontic infection	
Dialister: species are asaccharolytic obligately anaerobic Gram negative coccobacilli which have been consistently detected in endodontic infections.	• *Dialister pneumosintes* • *Dialister invisus*
Fusobacterium**:** is also a common member of endodontic microbiota.	*Fusobacterium nucleatum* • *Fusobacterium periodonticum*
Spirochetes: are highly motile, spiral-shaped, Gram negative bacteria with	• *Treponema denticola* • *Treponema sacranskii* • *Treponema parvum* • *Treponema maltophilum*

periplasmic flagella. All oral spirochetes fall into the genus *Treponema.* Prevalent species are:	• *Treponema lecithinolyticu*
Gram positive anaerobic rods: have also been found in endodontic microbiota like:	*Pseudoramibacter alactolyticus* • *Filifactor alocis* • *Actinomyces spp.* • *Propionibacterium propionicum* • *Olsenella spp.* • *Slackia exigua* • *Mogibacterium timidum* • *Eubacterium spp*
Gram positive cocci: that are present in endodontic infection:	• *Parvimonas micra* (previously called *Peptostreptococcus* micros *or* Micromonas micros) • *Streptococcus spp.* Which include: - *Streptococcus anginosus,* - *Streptococcus mitis,* - *Streptococcus sanguinis* • *Enterococcus faecalis.*
Other bacterial spp. which are present in low to moderate values include:	
***Campylobacter spp*.:** which are Gram negative anaerobic rods; common species are:	*Campylobacter rectus* • *Campylobacter gracilis*
• *Catonella morbic:* which is a saccharolytic obligate anaerobic Gram negative rod • *Veillonella parvula* • *Eikenella corrodens* • *Granulicatella adiacens* • *Neisseria mucosa* • *Centipeda periodontii* • *Gemella morbillorum* • *Capnocytophaga gingivalis* • *Corynebacterium matruchotii* • *Bifidobacterium dentium* • Anaerobic lactobacilli.	

Apart from these, several uncultivated phylotypes which can be unrecognized but play a role in pathogenesis of apical periodontitis, such as:	• *Dialister* oral clone BSO16 • *Migasphaera* oral clone BSO16 • Solobacterium • Olsenella • Eubacterium • Cytophaga • Lachnospiraceae oral clone 55A-34 • *Veillonella* oral clone BP 1–85 • Bacteroidetes oral clone XO 83 • *Prevotella* oral clone PUS 9.180 • *Eubacterium* oral clone BP 1–89 and • Lachnospiraceae oral clone MCE 7–60.

1.2.2.1.2 Persistent/secondary intraradicular infection:

A secondary intraradicular infection is an infection caused by microorganisms that were not present in primary infection but were introduced in the root canal system at some stage after intervention (Haapasalo *et al.* 2003; Chong 2017). Those microorganisms may have resistant to the chemomechanical procedures (persistent infection) or they may have invaded the canal through coronal leakage of the root filling (secondary infection) (Signoretti *et al.* 2011; Gomes and Herrera 2018).

These microorganisms have gained entry into the root canal system during treatment either between appointments, or at the end of the endodontic treatment, the most common being a breach of the aseptic chain during treatment or sometimes an insufficient root debridement during

initial endodontic treatment allows microbial species to reinstate the disease (Jayakodi *et al*. 2012; Anderson *et al*. 2013).

The persistence of those microorganisms within the root canal system after treatment is the major cause of root canal infections and endodontic treatment failure (Neelakantan *et al*. 2017). Persistent/secondary infections may cause several clinical conditions, including persistent exudation, persistent symptoms, inter-appointment flare-ups and treatment failure characterized by post-treatment apical periodontitis (Teles *et al*. 2013).

The microbiota found in endodontic failure is composed of a more restricted group of species with dominance of facultative bacteria when compared to primary infections (Siqueira and Rocas 2004; Siqueira and Rocas 2005). Adequately treated canals could harbor fewer than five species. While, inadequately treated teeth with unsatisfactory root filling may harbor 10 to 30 species, a number similar to that of primary infections (Gomes and Herrera 2018).

The microorganisms detected in root canal treated teeth associated with secondary intraradicular infections are shown in (Table 1.2) (Rocas and Siqueira 2008; Narayanan and Vaishnavi 2010; Torabinejad *et al.* 2015; Singh 2016).

The prevalence of certain species in teeth with post-treatment infection are *Enterococci*, *Streptococci*, *Lactobacilli*, *Actinomyces* and fungi (such as *Candida*). But the most prevalent and commonly detected species in a high proportion in persistent endodontic infections is *Enterococcus faecalis* (*E. faecalis)* which is a facultative gram-positive bacterium (Peciuliene *et al*. 2008; Ayer *et al*. 2015; Lee *et al*. 2017; Neelakantan *et al*. 2017). This bacterium has the potential to enter the dentinal tubules and withstand the action of all antimicrobial agents and medicaments that have been used in endodontics so far (Singh 2016).

Enterococcus faecalis can coexist with other species inside the root canal system, also it may exist as a single organism (Ramirez-Mora *et al*. 2018). Furthermore, *E. faecalis* has the ability to survive inside the root canal system for years due to its unique physiological characteristics and it can become active at a later stage, resulting in persistent endodontic infection (Jhajharia *et al*. 2015; Landete *et al*. 2018).

It was found that failed root canal-treated teeth are about nine times more likely to harbor *E. faecalis* than cases of primary infections (Rocas *et al*. 2004a).

Its presence in persistent/secondary infections ranges from 24% to 77% as confirmed by culture-based techniques (Hancock *et al*. 2001; Pinheiro *et al*. 2003; Adib *et al*. 2004; Gomes *et al*. 2004; Rocas *et al*. 2004a; Stuart *et al*. 2006). While in molecular techniques (polymerase chain reaction-PCR), the prevalence of *E. faecalis* was found to reach 67–77% (Rocas *et al*. 2004b; Siqueira and Rocas 2004; Sedgley *et al*. 2006; Stuart *et al*. 2006; Gomes *et al*. 2008), and reaching 90% in other studies (Dahlen *et al*. 2000; Fouad *et al*. 2005). On the other hand, Sundqvist *et al*. (1998) and Pinheiro *et al*. (2003) stated that *E. faecalis* has been documented to be the only organism (pure culture) present in root filled teeth with periradicular lesions.

Table 1.2 Bacteria causing secondary intraradicular infections. (Narayanan and Vaishnavi 2010; Torabinejad *et al*. 2015; Singh 2016).

MAIN GROUP OF BACTERIA SPECIES	
Gram negative anaerobic rods	• *Fusobacterium nucleatum* • *Prevotella spp.* • *Campylobacter rectus*
Gram positive bacteria	. *Streptococcus gordonii,* •*Streptococcus mitis* •*Streptococcus anginosus,* •*Streptococcus oralis* •*Lactobacillus paracasei* •*Lactobacillus acidophilus* •*Staphylococci* •*Enterococcus faecalis* •*Olsenella uli* •*Parvimonas micra* •*Pseudoramibacter alactolyticus* •*Propionibacterium spp.* •*Actinomyces spp.* •*Bifidobacterium spp.* •*Eubacterium spp.*

1.2.2.2 Extraradicular infection:

Extraradicular infection usually starts from an intraradicular infection which then spreads to the periradicular tissues through the apical foramen (Gomes and Herrera 2018).

Intraradicular microorganisms usually are limited within the root canal due to the defense barrier. But in some instances, those microorganisms can bypass this defense barrier and invade the extraradicular space leading to an extraradicular infection (Narayanan and Vaishnavi 2010). This could end up in the development of an acute apical abscess that is characterized by pus formation due to microbial invasion of the periapical tissues (Fouad 2017).

On the other hand, extraradicular infections may be found in untreated root canals, also can be found in post treatment apical periodontitis. Extraradicular infections are not as common as the intraradicular infections, however they can affect the end result of root canal treatment (Bhargava *et al*. 2015).

Species that have been demonstrated according to many studies as being involved in extraradicular infections are: Actinomyces species (*A. israelii, A. naeslundii, A. odontolyticus, A. viscosus), P.acnes, P. propionicum, P. gingivalis, P. intermedia, Prevotella oralis, P. micra, and F. nucleatum* (Siqueira and Rocas 2009; Narayanan and Vaishnavi 2010; Bhargava *et al*. 2015; Singh 2016).

1.3 *Enterococcus faecalis* bacteria:

Enterococcus faecalis, formerly classified as part of the group D *Streptococcus* system (Khan *et al*. 2018). The name “enterocoque” was first described by “Thiercelin”, from France, in a paper published in 1899;

the name was used to refer to the intestinal origin of this organism (Shenoy and Mala 2006).

Enterococcus faecalis is a Gram-positive spherical or ovoid cell 0.5 to 1 μm in diameter as shown in (Figure 1.1), that occurs singly, in pairs, or in chains of various lengths (Koch *et al*. 2004; Bhardwaj 2013; Zhou and Li 2015). Most strains are non-motile and non-hemolytic. They are facultative anaerobes, as they can grow in the absence as well as in the presence of oxygen (Shenoy and Mala 2006).

Various *E. faecalis* subtypes have been determined using molecular methods (PCR) amplification assays (Ke *et al*. 1999; Lebreton *et al*. 2014).

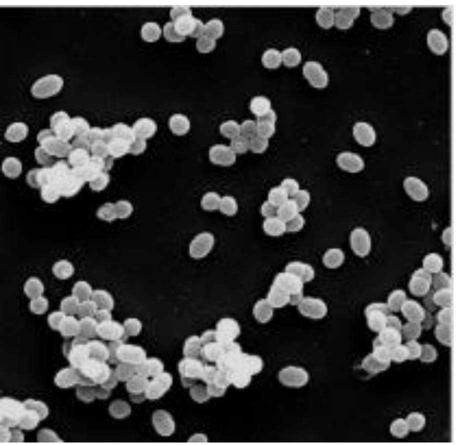


Figure 1.1 Colonies of *Enterococcus faecalis* bacteria (Zhou and Li 2015).

The commonest enterococcal species in the human normal flora is *E. faecalis* (Qin *et al*. 2010). They inhabit the gastrointestinal tract, oral cavity, and genitourinary tract of humans and animals. This species is regarded as transient bacteria in the oral cavity and its source could be food (Kayaoglu and ørstavik 2004; Zehnder and Guggenheim 2009). They are the causative agents of different conditions in humans, like urinary tract infection, septicemia, endocarditis, gastroenteritis, cholecystitis, burn

wounds infection, and infection of indwelling foreign devices (Souto *et al*. 2008).

Enterococcus faecalis can cause life-threatening infections in humans, especially in the nosocomial (hospital) admitted patients, due to the high levels of antibiotic resistance found in *E. faecalis* is the cause of its virulence (Ryan and Ray 2004). *Enterococci* are considered one of the top three nosocomial bacterial pathogens. *E. faecalis* causes up to 90% of *enterococcal* infections in humans (Kayaoglu and Qrstavik 2004; Olawale *et al*. 2011). In dentistry, *Enterococcus* species, in particular *E faecalis* is blamed as the causative agent of chronic periodontitis and failed root canal treatments involving chronic apical periodontitis (Wang *et al*. 2012a; Khalifa *et al*. 2016; Gomes and Herrera 2018; Dioguardi *et al*. 2019).

1.3.1 Physiology of *Enterococcus faecalis*:

Enterococcus faecalis is a non-motile microbe that can ferment glucose without gas production, also it does not produce a catalase reaction with hydrogen peroxide (Stuart *et al*. 2006). Although this bacterium is catalase-reaction negative, it may show a weak positive reaction to catalase under certain conditions, furthermore, it may rarely produce a pseudo-catalase reaction in cases where it is grown on blood-containing media (blood agar). However, since this reaction is weak, it can be neglected (Frankenberg *et al*. 2002).

This microorganism which is a facultative anaerobe, capable of using different kinds of energy sources with the metabolic end product always being lactic acid. It typically grows in a temperature range of 10°C to 45°C but has an optimal growth at 35°C (Araújo and Luces 2015).

Enterococci are known to be highly resistant and capable of withstand extreme conditions. *E. faecalis* can resist oxidative stress, disinfectants, detergents, heavy metals, bile salts, ethanol, sodium azide, and resist

desiccation and persist for weeks or even up to months (Kramer *et al*. 2006; Howie *et al*. 2008).

Enterococcus faecalis can persist in tissues because they can survive tough conditions due to capacity to form biofilm making them more resistant to phagocytosis, antibodies and antimicrobial agents (Vasudeva *et al*. 2017). Moreover, *E. faecalis* can survive in highly acidic (pH 4.0) and alkaline (pH 9.6) conditions. In addition, they have the potential to hydrolyse leucin-pyrrolidonyl-β-naphthylamide (PYR) and esculin in the presence of 40% bile salts (Facklam *et al*. 2002).

Enterococci can grow at 10ºC and 45ºC in 6.5% NaCl broths and survive at 60ºC for 30 minutes. Due to all of these unique characteristics of *E. faecalis*, this bacterium can survive in root canal infections, where nutrients are limited and there are no means of escape from root canal medicaments (Bhardwaj 2013).

1.3.2 Role of *Enterococcus faecalis* in pathogenesis of endodontic infection*:*

Enterococcus faecalis is a normal inhabitant of the oral cavity, but the prevalence of *E. faecalis* is elevated in oral rinse samples from patients receiving initial endodontic treatment, those midway through treatment, and patients receiving endodontic retreatment when compared to those with no endodontic history (Wang *et al*. 2012a; David and Kishor 2011).

Enterococcus faecalis is retrieved from various forms of periradicular disease including primary endodontic infections and persistent infections (Sassone *et al*. 2007; Ozok *et al*. 2012; Bouillaguet *et al*. 2018). In primary endodontic infections, *E. faecalis* is found in asymptomatic chronic periradicular lesions significantly more than in acute periradicular periodontitis or acute periradicular abscesses. *E. faecalis* is found in 4 to 40% of primary endodontic infections. While it has a predominant role in

the pathology of persistent periradicular lesions following root canal treatment (Rocas *et al*. 2004a; Stuart *et al*. 2006; Williams *et al*. 2006; Vineet and Nayak 2016).

Several studies have demonstrated the predominance of *E. faecalis* in endodontic failed root canals (Souto and Colombo 2008; Wang *et al*. 2012a; Zhang *et al*. 2012; Martina *et al*. 2013; Ravinanthanan *et al*. 2018).

Enterococcus faecalis can inhabit dentinal tubules, isthmus, rami, lateral and accessory canals. *E. faecalis* can survive on serum components from the dentinal fluid as the root canal is a medium that is poor in nutrients (George *et al*. 2005; Madhavan and Muralidharan 2015). This bacterium has the ability to survive in root canal system as a single organism without the support of other bacteria and is small enough to efficiently invade and live within the dentinal tubules (Shaik *et al*. 2014). Consequently, *E. faecalis* are commonly the target microorganism during assessment of antibacterial efficacy of irrigating solutions, intracanal medications and preparation techniques (Tendolkar *et al*. 2003; Estrela *et al*. 2008; Vidana *et al*. 2011).

The wide range of *E. faecalis* prevalence among different studies can be attributed to various identification techniques, geographic differences, or sample size (Baumgartner *et al*. 2004; Fouad *et al*. 2005). The high survival and virulence of *E. faecalis* can be attributed to its ability to compete with other microorganisms, invade dentinal tubules, and resist nutritional deprivation (Staurt *et al*. 2006). The ability to endure starvation is a beneficial survival characteristic which assist *E. faecalis* to persist more than others until there are nutrients from the local environment, such as serum-type fluid that may pour into the canal space over time (Teles *et al*. 2013).

Enterococcus faecalis can remain viable for up to twelve months without any nutrients (Sedgley *et al*. 2006). It has high antimicrobial

resistance and possesses ability to persist in a changing environment, that is why it can survive in root canal and cause re-infection. *E. faecalis* can adhere to root canal walls, forming a biofilm, which helps it to resist destruction by enabling the bacteria to become 1000 times more resistant to phagocytosis, antibodies, and antimicrobials than non-bio film-producing organisms (Stuart *et al*. 2006).

A study by (Wang *et al*. 2012a) showed that *E. faecalis* is more commonly found in root canals with unsatisfactory obturation than in those having satisfactory obturation (19/58 vs. 3/58). This can be explained by the fact that the inadequately obturated root canal provides more space and nutrients than the well-obturated canals, furthermore, the available space can ensure a facultative anaerobic environment. Additionally, inadequate cleaning and shaping might leave infected debris behind. *E. faecalis* can persist inside the small canals of apical ramifications or in the space between the root filling and canal wall. In contrary, well-obturated canals keep an obligate anaerobic environment that does not favor the survival and growth of *E. faecalis*.

1.3.3 Virulence factors associated with *Enterococcus faecalis:*

For any bacterium to be pathogenic, it should adhere, grow, and invade the host. Then, it must resist the host defense mechanisms, compete with other bacteria, and produce pathological changes. Because of the virulence factors owned by *E. faecalis*, it has the required conditions to initiate an endodontic infection and maintain an inflammatory response (Kayaoglu and Ørstavik 2004).

Virulence factors of *Enterococcus faecalis* include:

- Cytolysin and proteolytic enzymes (gelatinase and serine protease),
- Adhesins; aggregation Substance (AS), Enterococci surface protein (Esp), collagen adhesion protein (Ace), antigen A (EfaA),

- capsular and cellular wall polysaccharides Lipoteichoic acids (LTA) (Thurlow *et al*. 2009; Zoletti *et al*. 2011).

Once contamination of the root canal with *E. faecalis* takes place, it colonizes the dentinal walls by adhering to the mineral part, possible by LTA, and to the collagen by AS and other surface adhesins (Kayaoglu and Qrstavik 2004).

Enterococcus faecalis may alter the adhesin expression under stressful conditions such as deficiency of nutrients, other bacterial toxins, or endodontic medicaments with the help of adhesive moieties (Halkai *et al*. 2012). Furthermore, additional mechanism through which *E. faecalis* can survive, is by LTA, which promotes the resistance of the bacterium against a variety of lethal conditions (Kayaoglu and Qrstavik 2004).

In a mixed infection, *E. faecalis* uses its cytolysin to inhibit the growth of other bacteria, thus facilitating the predominance of *E. faecalis* (Mallick *et al*. 2014). Hyaluronidase is an enzyme used by *E. faecalis* to produce the energy it needs from the hyaluronan present in the dentin (Kayaoglu and Ørstavik 2004). The enzyme hyaluronidase helps the spread of bacteria as well as their toxins through host tissues.

Gelatinase is an enzyme that is responsible for bone resorption and degradation of dentin organic matrix, hence it plays a major role in the etiology of periapical inflammation. The adherence of *E. faecalis* to dentin is facilitated by serine protease which breaks the peptide bonds. On the other hand, bacteriocins like AS-48 suppresses the growth of other organisms (Shenoy and Mala 2006; Mallick *et al*. 2014).

1.3.4 Antimicrobial resistance associated with *Enterococcus faecalis*:

Bacterial strains possessing genes responsible for antibiotic resistance in endodontic infections have been found. This explains why persistent

infections occur when the same antibiotic is used topically in the canal, these resistant strains can therefore survive. If a persistent infection caused by antibiotic resistant strains becomes acute, it is ineffective to use systemic antibiotics (Chong 2017).

Enterococcus faecalis possesses the ability to be resistant to virtually all clinically useful antibiotics (Hollenbeck and Rice 2012). This will assist the organism to survive in an environment in which antimicrobial agents have been applied (Mallick *et al*. 2014). Hence, the clinical importance of *E. faecalis* is due to its antibiotic resistance, which highly increases the risk of colonization and infection (Zapun *et al*. 2008).

Resistance is the ability of a microorganism to survive in the presence of high concentrations of an antimicrobial agent or the combination of antimicrobial medications (Kudiyirickal and Ivancakova 2008). These bacteria own intrinsic resistance to multiple antibiotics, additionally they have the ability to rapidly develop antibiotic resistance through gene transfer (Lins *et al*. 2013). Intrinsic resistance is unique of a particular bacterium and it relies on inherent anatomy of the microorganism. It is defined as the resistance to a specific antibiotic to which all members of a species are resistant. While acquired resistance represents a new heritable variation in the bacterial DNA (Mullany 2014).

Acquired resistance is achieved by either a mutation in the existing DNA or development of a new gene, via the transfer of plasmids and transposons. *E. faecalis* has the advantage of having an intrinsic resistance to many antimicrobial agents which gives them a cumulative advantage for further development of genes encoding high-level resistance to aminoglycosides, penicillin, tetracycline, microlides chloramphenicol, and now vancomycin (Mallick *et al*. 2014; Chong 2017).

Enterococcus faecalis have intrinsic resistance to clindamycin, quinupristin and dalfopristin by expression of the chromosomal gene named (Lsa) gene (Hollenbeck and Rice 2012). Complete or relative resistance to β-lactams antibiotics is a unique feature of *E. faecalis* (Cetinkaya *et al*. 2000). Consequently, the treatment of infections caused by *E. faecalis* is usually complicated, especially for severe infections, which due to intrinsic resistances require a combination of antibiotics (Zapun *et al*. 2008).

1.3.5 Biofilm formation by *Enterococcus faecalis*:

Enterococcus faecalis resist various intracanal treatment procedures. This is attributed for their ability to penetrate dentinal tubules, possess virulence factors and because of biofilm formation (Saber and El-Hady 2012).

Biofilms are multicellular microbial communities that adhere to surfaces and interfaces. The formation and maintenance of biofilms is dependent on the production of extracellular substances including proteins and exopolysaccharides that constitute the extracellular matrix (Flemming and and Wingender 2010).

These extracellular matrices secure the bacteria together in a multicellular community. In the root canal environment, *E. faecalis* bacterium plays an important role in bacterial biofilm formation, and therefore *E. faecalis* biofilms are considered to be an appropriate model for testing novel antimicrobial treatments (Rosen *et al*. 2016).

Enterococcus faecalis in root canals mainly exists as a biofilm by adhering to the root canal walls, accumulating and forming communities that are 1000 times more resistant to antimicrobials, antibodies and phagocytosis than isolated planktonic organisms (Ran *et al*. 2015).

Biofilm mode of growth is advantageous for *E. faecalis*, as they form three-dimensional structured communities with fluid channels for transport of substrate, waste products, and signal molecules. Biofilm formation in root canals is probably initiated sometime after the first invasion of the pulp chamber by planktonic oral microorganisms after some tissue breakdown (Jhajharia *et al*. 2015).

However, biofilm formation is generally limited by nutritional conditions, and for gram-positive bacteria, including *E. faecalis*, carbohydrate metabolism may significantly affect biofilm formation (Pillai *et al*. 2004).

Glucose and maltose are the most important carbohydrates affecting biofilm formation. However, the physicochemical properties of *E. faecalis* biofilms were found to modify according to the prevailing environmental and nutrient conditions. *E. faecalis* under nutrient rich environment produces typical biofilm structures with characteristic surface aggregates of bacterial cells and water channels (Breton *et al*. 2005; Jhajharia *et al*. 2015; Keogh *et al*. 2018).

Enterococcus faecalis have the ability to form a calcified biofilm on root canal dentine and it may be a factor that contributes to their persistence after endodontic treatment through a bacterial-induced apatite reprecipitation on mature biofilm (Kishen *et al*. 2006).

The stages of structural organization of biofilm, the composition and activities of the colonizing microorganisms in various environments may be different, although the establishment of a micro-community on a surface seems to follow essentially the same series of developmental stages (Estrela *et al*. 2009).

The development of *E. faecalis* biofilm on the root canal dentin involves three stages as follows:

- Stage 1: Microcolonies are formed as *E. faecalis* cells adhere on the root canal dentin surface
- Stage 2: Bacterial-mediated dissolution of the mineral fraction from the dentin substrate leads to localized increase in the calcium and phosphate ions causing mineralization (or calcification) of the *E. faecalis* biofilm
- Stage 3: Due to this interaction of bacteria and their metabolic products on dentin, *E. faecalis* biofilm is mineralized. (Hubble *et al*. 2013; Jhajharia *et al*. 2015)

1.4 Management of failed endodontic treatment:

Any method used to disinfect the root canal should be effective in reaching and eradication of microbes as much as possible from all areas of the root canal system (coronal, middle and apical third) (Coldero *et al*. 2002; Demiryurek *et al*. 2014).

The essential constituents of the of endodontic infection are: host defense mechanism, systemic antibiotic administration (only in special indications), endodontic therapy which includes three important steps to control infections of the root canal system; chemo-mechanical preparation (instrumentation and irrigation), locally applied intracanal medicaments between appointments (in cases of retreatment and in infected roots), and lastly, obturation of the root canal and coronal restoration (Haapasalo *et al*. 2003; Mohammadi 2010).

Bacteria persisting in the dentinal tubules represent the major cause of apical periodontitis. Thus, successful root canal treatment should achieve bacteria-free root canal system to ensure proper healing of the periradicular

tissues (Ooi *et al*. 2019). *Enterococcus faecalis* species is predominantly retrieved in persistent endodontic infections and increases the risk for failed endodontic infection (Siqueira and Rocas 2008; Demiryürek *et al*. 2014).

Studies are focused around investigating successful means to eradicate *E. faecalis* in the root canal system (Stuart *et al*. 2006).

The chemomechanical preparation of the root canal aims at preventing endodontic infection, but multiple studies demonstrated that even after chemomechanical preparation, *E. faecalis* could persist within the complex anatomy of the root canal system due to the many inaccessible areas which supply an ideal environment for microorganisms to survive and proceed in their pathological process (ValeraI *et al*. 2009; Luddin and Aly 2013; Demiryürek *et al*. 2014; Borzini *et al*. 2016).

Peters *et al*. (2001) reported that 35% or more of the root canal surfaces remained uninstrumented, in spite of utilization of modern nickel titanium instruments. *Enterococcus faecalis* enters a non-cultivable state during root canal treatment and survives the chemo-mechanical procedures that are proven to be bactericidal. After return to a favorable environment, *E. faecalis* returns into a cultureable state (Demiryürek *et al*. 2014; Henriques *et al*. 2016).

Additionally, *E. faecalis* has the ability to adhere to dentin and efficiently invades dentinal tubules and penetrates deep up to 400 µm in vitro, therefore, bypassing the effects of chemo-mechanical preparation during the root canal (Hegde 2009; Al-Nazhan *et al*. 2014; Pandey *et al*. 2018). Many studies reported that instrumentation and antibacterial irrigation using sodium hypochlorite (NaOCl) can reduce bacteria in 50% to 75% of infected root canals at the conclusion of the first treatment visit, while the rest of the root canals still harbor recoverable bacteria (Bystrom and Sundqvist 1983; Peters *et al*. 2002; Nair *et al*. 2005).

Authors demonstrated that *E. faecalis* bacteria can resist the action of NaOCl even at concentrations higher than 5% (Dioguardi *et al*. 2018; Dioguardi *et al*. 2019). This is attributed to many factors; the penetration power of NaOCl in the dentinal tubules has been found to be only 300 µm, additionally, the high surface tension of NaOCl reducing its penetration into the dentinal tubules, minimizing its antibacterial efficacy (Priyank *et al*. 2017). Thus, intracanal medicament between appointments is recommended for disinfection and for the purpose of assurance of further elimination in microbiota in the root canal system (Marickar *et al*. 2014; Somanath *et al*. 2015; Mahendra *et al*. 2016).

1.4.1 Intracanal medicaments:

Intracanal medicaments are defined as antiseptic agents in the chemical form applied to the walls of the root canals in order to eradicate microorganisms present before or even following instrumentation and irrigation of the root canal system between appointments (Bansal and Jain 2014). Selection of intracanal medicaments relies on the diagnosis of the type of endodontic infection, a wide orientation of the types of causative microorganisms, and their mechanisms of growth and survival (Parhizkar *et al*. 2018).

1.4.1.1 Ideal properties of intracanal medicaments:

The ideal properties and the requirements of an intracanal medicament are: (Grossman 1998; Garg and Garg 2013; Pal *et al*. 2019).

- It should be germicidal and fungicidal.
- It should be non-irritant.
- It should have a long duration of antibacterial effect.
- It should be effective in any circumstances like presence of blood, serum and protein derivatives of tissue.

- It should penetrate the tissue deeply.
- It should cause degradation of microbial biofilm and residual organic material.
- It should be biocompatible (not induce any cell-mediated immune response).
- It should not derange the tooth structure (the microhardness of the tooth).
- It should be easily applied into the root canal.
- It should prevent coronal microleakage and not diffuse through the temporary restoration.
- It should have low surface tension.
- It should not cause crown discoloration.
- It should be inexpensive.

1.4.1.2 Objectives of intracanal medicaments:

Intracanal medicaments used to improve the predictability and prognosis of endodontic treatment. They are used in endodontic therapy in order to:

- Eradicate any persisting viable bacteria in the root canal system that have survived the chemomechanical preparation processes (instrumentation and irrigation).
- Reduce periradicular inflammation and hence reduce pain.
- Eliminate apical exudate if it is present.
- Prevent or arrest inflammatory root resorption if it is present.
- Prevent reinfection of the root canal system by acting both as a chemical and physical barrier if the provisional restoration cracks. (Walton 1998; Kohle 2010; Murvindran and Raj 2014).

1.4.1.3 Classification of intracanal medicaments:

Because of a variety of usage of intracanal medicament, there is diversity in agents.

According to Weine (1995) intracanal medicaments can be classified as:

1. Phenol and related compound:
    - Eugenol
    - Camphorated paramonochlorophenol
    - Metacrecyacetate
    - Cresol
    - Thymol
2. PBSC:
    - Penicillin
    - Bacitracin
    - Streptomycin
    - Caprylate
3. Sulphonamides:
4. Corticosteroid-antibiotic combinations
5. Calcium hydroxide

1.4.1.4 Effect of different intracanal medicaments on the *Enterococcus faecalis*

a. <u>Calcium hydroxide (CH):</u>

Calcium hydroxide is used as an intracanal medicament, however its efficacy against *E. faecalis* is questionable (Lana *et al*. 2009; Murvindran and Raj 2014). Calcium hydroxide due to its high pH (12. 9) has been used as an intracanal medicament but, it is not equally effective against all species of microorganisms found in the root canal system (Gomes *et al*. 2002).

Waltimo *et al*. (2005) found that CH dressing between appointments could not demonstrate the desired results in disinfection of the root canal system and in treatment outcome. Although, CH has an alkaline pH and a broad anti-microbial spectrum, *E. faecalis* has been found to be resistant to the actions and antimicrobial effect of CH, as it persisted in the dentinal tubules (Wang *et al*. 2012b; Shaik *et al*. 2014; Dianat *et al*. 2015). This has drawn so much research in endodontics looking for an alternative intracanal medicament.

b. <u>Chlorhexidine gluconate (CHX):</u>

Chlorhexidine gluconate has been used in endodontics both as an irrigant and intracanal medicament (Gomes *et al*. 2013). It is effective against gram-positive, gram-negative, aerobic, and anaerobic organisms, as well as the *Candida* species. It is effective against microorganisms that are resistant to CH (Athanassiadis *et al*. 2007; Vaghela *et al*. 2011).

Several studies (Schafer and Bossmann 2005; Gomes *et al*. 2006; Sinha *et al*. 2013) showed that 2% CHX gel is more effective than CH paste against *E. faecalis*. However, chlorhexidine is unable to dissolve pulp tissue, therefore, debris will remain on canal walls, obstructing the dentinal tubules (Mittal *et al*. 2012). Moreover, CHX are less soluble substances and they can leave residues on the root canal surfaces after viscous irrigants, affecting the sealing ability of the sealer and obturation material (Lambrianidis *et al*. 2006).

Estrela *et al*. (2008) found that CHX showed low efficacy to eradicate *E. faecalis* when assessed by PCR or culture techniques. This was confirmed by (Krishna *et al*. 2012; Lucena *et al*. 2013; Vasudeva *et al*. 2017; Yadav *et al*. 2018) who showed that CHX were effective in decreasing the viability of *E. faecalis* but has not the efficacy to eradicate this bacterium completely. However, as it lacks property of tissue solubility

and possesses cytotoxic effect, some individuals may also be allergic to it, CHX cannot be used as a routine medicament (Ferraz *et al*. 2001).

1.4.2 The role of antibiotics in failed endodontic treatment:

For many decades, antibiotics have been prescribed in various fields of medicine and dentistry for the treatment of bacterial infections. In dentistry, systemic or local antibiotics may be used during root canal treatment (Sabharwal *et al*. 2019).

Systemic antibiotics can be used as an adjuvant clinical treatment in some surgical and nonsurgical endodontic cases, however, their use may be associated by adverse systemic effects, such as allergic reactions, toxicity, various side effects and the development of resistant strains of microbes (Mohammadi *et al*. 2018).

Local application of antibiotics in the root canal in the form of intracanal medicaments has been suggested as an alternative to prevent the potential risks of adverse systemic effects of antibiotics. It represents an effective method for delivering the drug in teeth without blood supply due to necrotic pulps or pulp-less status (Mittal and Jain 2014; Shaik *et al*. 2014; Prasada *et al*. 2016).

1.4.2.1 Advantages and disadvantages of locally used antibiotic agents:

The advantages of locally used antibiotic agents include:

- Efficient and predictable disinfection.
- Application of high drug concentrations at the local site.
- Reduction of systemic complications of antibiotic medications.

While, the disadvantages of locally used antibiotic agents are:

- Possible emergence of bacterial resistant strains (antimicrobial resistance).

- Allergic reactions.
- Inhibition of angiogenesis.
- Tooth staining or discoloration.

(Bansal and Jain 2014; Balasubramaniam and Jayakumar 2017)

1.4.2.2 Locally used antibiotic agents in endodontics:

In 1951, Grossman was first to use local antibiotics in endodontics in the form of poly-antibiotic paste, known as PBSC (penicillin, bacitracin, streptomycin, and caprylate sodium) (Ashish *et al.* 2015).

Penicillin was supposed to target Gram-positive organisms, bacitracin was used for penicillin-resistant strains, while streptomycin was targeting Gram-negative organisms, lastly, caprylate sodium was directed against yeasts. Those compounds were all suspended in a silicone vehicle (Murvindran and Raj 2014).

The clinical evidence concluded that the paste was therapeutically effective, but the composition was ineffective against anaerobic species that are now considered the predominant organisms responsible for endodontic diseases (Patel 2015b).

In 1975, the USA Food and Drug Administration (FDA) banned PBSC in endodontics because of the risks of sensitization and allergic reactions caused by penicillin (Bansal and Jain 2014).

1.4.2.3 Types of locally used antibiotic agents in endodontics:

The type of locally used antibiotic agents in endodontics as intracanal medicaments are:

- Ledermix Paste
- Odontopaste
- Septomixine Forte
- Sulfonamides
- TAP - Triple Antibiotic Paste

- **Ledermix paste:**

Ledermix is used both as pulp capping agent and as an intracanal medicament (**Athanassiadis *et al.* 2007**). In 1960, Schroeder and Triadan developed Ledermix which was made commercially available by Lederle Pharmaceuticals in 1962. Ledermix paste contains an antibiotic demeclocycline-HCl 3.2% and a corticosteroid (triamcinolone acetonide 1%), in a polyethylene glycol base (Ahmed and Abbott 2012).

The corticosteroid minimizes periapical inflammation and instantly relieve the pain, while the antimicrobial action is performed by antibiotics (Kundabala *et al.* 2014). However, this paste could not be advised for use against endodontic microorganisms due to their inadequate spectrum of activity (Parhizkar *et al.* 2018). Furthermore, Ledermix paste causes crown discoloration being used as an intracanal medicament (Kim *et al.* 2000).

- **Odontopaste**

Odontopaste is a zinc oxide-based root canal paste with clindamycin hydrochloride 5% and triamcinolone acetonide 1%, formulated and developed in Australia (Eftekhar *et al.* 2013). The steroid part, triamcinolone acetonide, can temporarily reduce inflammation and postoperative pain (Balasubramaniam and Jayakumar 2017). This antibiotic provides a bacteriostatic activity in addition to benefits of a zinc oxide paste.

However, Odontopaste cannot eliminate all of the bacteria within the root canal (Eftekhar *et al.* 2013). Moreover, Plutzer *et al.* (2018) stated that Odontopaste as well as Ledermix have no significant effect on the *E. faecalis* biofilm.

- **Septomixine forte** :

Septomixine forte paste contains dexamethasone, halethazole tartrate, neomycin sulfate, polymyxin B sulfate, and tyrothricin (Athanassiadis *et al*. 2010).

Neomycin is bactericidal against Gram-negative bacilli but it is ineffective against Bacteroides and related species, as well as against fungi. Polymyxin B sulphate is ineffective against Gram-positive bacteria (Murvindran and Raj 2014).

Tang *et al*. (2004) demonstrated that a routine one-week local application of Septomixine forte could not effectively inhibit the residual intracanal bacterial growth between appointments. However, it is no longer recommended as an intracanal medicament because the antibiotics (neomycin and polymyxin B sulfate) are ineffective for use against endodontic bacteria due to their improper spectrum of antibacterial activity (Bansal and Jain 2014).

Chu *et al*. (2006) showed that there were no significant differences in the number of bacterial growth between Septomixine forte and Ledermix.

- **Sulfonamides:**

Sulfanilamide and sulfathiazole were initially used as root canal medicaments, but now are no longer used as they tend to cause a yellowish discoloration of the teeth (Bansal and Jain 2014).

- **Triple antibiotic paste (TAP):**

Triple antibiotic paste contains both bactericidal (metronidazole, ciprofloxacin) and bacteriostatic (minocycline) agents. This combination is commercially available as 3-MIX MP (Vijayaraghavan *et al*. 2012).

TAP was first introduced for the purpose of regeneration and revascularization protocol and the treatment of open apex teeth with necrotic pulp. TAP was developed by Hoshino and colleagues in 1996, who studied the efficacy of the paste on the removal of microorganisms from the root canals (Mohammadi *et al*. 2018; Parhizkar *et al*. 2018), while it was first clinically applied by Sato *et al*. in 1996 (Murvindran and Raj 2014).

Metronidazole is a nitroimidazole compound that exhibits a broad spectrum of activity against protozoa and anaerobic bacteria. Minocycline is a semisynthetic derivative of tetracycline with a similar spectrum of activity (Parasuraman and Muljibhai 2012). Metronidazole exerts bactericidal effects through penetrating bacterial cell membrane and binding to the DNA, disrupting the helix structure, resulting in a very rapid death (Mohammadi *et al*. 2018).

Minocycline is bacteriostatic which demonstrates antibacterial effects towards gram-positive and gram-negative bacteria. It also causes an elevation in the amount of interleukin-10, which is an inflammatory cytokine (Parhizkar *et al*. 2018).

Ciprofloxacin, which is a synthetic fluoroquinolone has a rapid bactericidal action and exhibits high antimicrobial activity against gram-negative bacteria, but low activity against gram-positive bacteria (Parhizkar *et al*. 2018). It inhibits the bacterial DNA gyrase enzyme, which nicks the double stranded DNA, producing negative supercoil and then reseals the nicked end. The bactericidal effect may be attributrde to the digestion of DNA by exonucleases which are released in response to DNA damage. Most of the anaerobic bacteria are resistant to ciprofloxacin (Murvindran and Raj 2014).

Although TAP is an established antibiotic paste, but it has its own drawbacks; TAP is radiolucent (Bansal and Jain 2014), and another concern of the antibiotic paste is that it may cause bacterial resistance (Slots 2002; Huang 2008). Although several studies showed its efficacy to reduce *E faecalis,* but it lacks the ability to eradicate this bacterium completely (Sabarathinam *et al*. 2018). Mozayeni *et al*. (2014), Mehta *et al*. (2017) and Ravi (2017) showed that TAP has better antibacterial efficacy against *E. faecalis* than CHX gel and CH, but TAP couldn't eradicate it completely.

Higher doses of TAP could produce undesired results on the stem cells. TAP should be used in the safest possible concentration (1 mg/mL) since even at low concentrations, TAP has undesirable effects on the proliferative capacity and mineralized matrix formation of dental pulp cells and apical papilla cells (Parhizkar *et al*. 2018).

Another drawback of TAP that it affects the microhardness of radicular dentine, as in vitro studies by (Eliades 1994; Yassen *et al*. 2015) demonstrated that TAP due to its acidic pH 2.9 can highly demineralize the dentine surface and will end up in a reduction in radicular dentine microhardness, also changes in the chemical structure of the superficial dentine can be seen when compared with calcium hydroxide.

In addition, research in attenuated total reflection-fourier transform infrared spectroscopy measurements indicated that 1 g/mL TAP showed severe reduction in microhardness of the root and demineralization (Prather *et al*. 2014; Nerness *et al*. 2016). On another hand, Elgendy and Nagy (2015) showed that TAP has an adverse effect on the fracture resistance of root canal dentine, especially when TAP is compared with chlorhexidine as an intracanal medicament.

Furthermore, the crown discoloration is another disadvantage of TAP due to minocycline (Dodd *et al*. 1998; Lenherr *et al*. 2012; Kirchhoff *et al.* 2015; Jagdale *et al*. 2018). Therefore, studies have proposed removing minocycline in a double antibiotic paste (DAP), keeping only metronidazole and ciprofloxacin (Kamocki *et al*. 2015; Gokturk *et al*. 2016), or substituting the minocycline with an alternative antibiotic, such as clindamycin, cefaclor, or amoxicillin to form modified triple antibiotic paste (MTAP) (Park *et al*. 2015; Changlani *et al*. 2017; Karczewski *et al*. 2018).

- **Modified triple antibiotic paste (MTAP):**

As mention above, modified triple antibiotic paste is a modification of TAP by replacing minocycline with clindamycin to avoid tooth discoloration and potential demineralization from minocycline. Clindamycin is a narrow-spectrum antibiotic and was found to be effective against different anaerobic bacteria isolated from primary endodontic infections (Karczewski *et al*. 2018).

A study by (Algarni *et al*. 2015) demonstrated that MTAP had significantly reduced biofilm formation by *E. faecalis* but it could not eradicate it completely with a similar efficacy to TAP.

Another drawback; as in vitro observations by (Prather *et al*. 2014) reported that MTAP as well as TAP and DAP showed significant reductions in microhardness of radicular dentine when compared to untreated control group. Additionally, a study by (Sipert *et al*. 2019) demonstrated that MTAP showed cytotoxic effects against apical papilla cells.

1.5 Nitrofurantoin:

Nitrofurantoin (Furadantin, Macrobid, Macrodantin) sold under the name Macrobid among others (McOsker and Fitzpatrick 1994). Nitrofurantoin with its molecular structure, as shown in (Figure 1.2), is a well-known antibacterial agent widely used as an oral treatment for treatment of urinary tract infections (UTIs). This drug is on the World Health Organization Model List of essential medicines and belongs to the Class IV drugs according to the Biopharmaceutics Classification System (BCS) (Zhang *et al*. 2019).

Nitrofurantoin is a synthetic nitrofuran compound (Sargın *et al*. 2012), it was created from furan and an added nitro group and a side change containing hydantoin (Asadi Karam *et al*. 2019). [N-(5-Nitro-2-Furyldine)-1-aminohydantoin] has the chemical formula $C_8H_6N_4O_5$ (Ingalsbe *et al*. 2015).

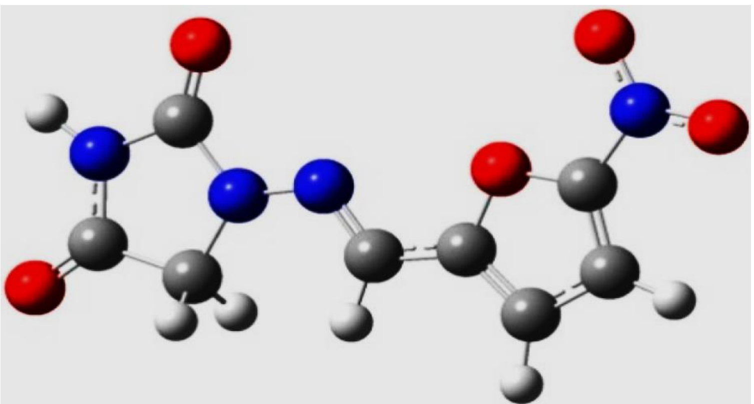


Figure 1.2 Molecular structures of nitrofurantoin (Zhang *et al*. 2019)

1.5.1 Medical Uses:

Nitrofurantoin was approved by the FDA in 1953 and is used for the treatment of uncomplicated lower urinary tract infections, chronic and recurrent infections (Butt *et al*. 2004; Qiao *et al*. 2013; Asadi Karam *et al*. 2019; Gardiner *et al*. 2019).

The efficacy of nitrofurantoin in treating UTIs combined with a low rate of bacterial resistance to this agent makes it one of the first-line agents for treating uncomplicated UTIs as recommended by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases (Gupta *et al*. 2011).

Besides, more recently, several major guidelines have implemented the use of nitrofurantoin as the first-line therapy for the treatment of uncomplicated lower UTIs. The high resistance to newer antibiotics in addition to increasing prevalence of extended-spectrum beta-lactamase (ESBL) producing bacteria have led to the recommendation for using nitrofurantoin (Ghazvini *et al*. 2019).

1.5.2 Antibacterial activity:

Nitrofurantoin is effective against most Gram-positive and Gram-negative organisms (Asadi Karam *et al*. 2019). It was demonstrated that it has good efficacy in controlling infections caused by the following microorganisms: *Enterococcus faecalis, E. coli, Staphylococcus saprophyticus, Coagulase negative staphylococci, Staphylococcus aureus, Streptococcus agalactiae, Citrobacter species, Klebsiella species* (CLSI 2012; Qiao *et al*. 2013).

Nitrofurantoin is the drug of choice as a therapy of infections caused by multidrug-resistant pathogens (Iravani *et al*. 1999; Munoz-Davila 2014; Shakti and Veeraraghavan 2015). In a study including 300 isolates of *Enterococcus*, none of the 300 isolates showed resistance to nitrofurantoin, including *E. faecalis* (Zhanel *et al*. 2001). Various studies confirmed that nitrofurantoin is highly effective against *E. faecalis* (Abdulla and Abdulla 2006; Rahbar *et al*. 2007; Toner *et al*. 2016).

1.5.3 Mechanism of action:

Nitrofurantoin's mechanism of action was poorly understood since its discovery in the 1940's. Nitrofurantoin uses several mechanisms to achieve an antimicrobial effect (Squadrito and Portal 2019). The mechanism of action is unique and complex. Nitrofurantoin possesses bacteriostatic and bactericidal effects, it kills bacteria through DNA damage, RNA damage, protein damage, inhibits and damage cell wall protein synthesis, as shown in (Figure 1.3) (Blass 2015).

Figure 1.3 Mechanism of action of nitrofurantoin. Bacterial Nitroreductase converts nitrofurantoin to the corresponding nitroso compound (red) which kills bacteria via DNA damage, RNA damage, and proteins damage (Blass 2015).

Nitrofurantoin interferes with bacterial carbohydrate metabolism by inhibiting acetylcoenzyme A. It is bacteriostatic at low concentrations (5-10 mg/mL) and is bactericidal at higher concentrations (Ebadi 2008).

Nitrofurantoin exhibits greater effects on bacterial cells than mammalian cells because bacterial cells activate the drug faster than do mammalian cells. It is not well understood which of the actions of nitrofurantoin is primarily causing its bactericidal effect. The broad spectrum of mechanisms of action of nitrofurantoin is contributing to the low resistance rate to its antibacterial effects, as the drug damages many major processes essential and vital to the bacterial cell (Batabyal 2018).

This explains the absence of bacterial resistance, bacterial resistance emerges as a result of natural selection and mutation. The mechanism behind development of bacterial resistance to an antimicrobial agent such as penicillin, ciprofloxacin, metronidazole, or any other ordinary antibiotic, involves only one mechanism of action that should be overcome. While for nitrofurantoin, however, mutations in bacterial genes responsible for producing resistance to at least the four mechanisms listed must all be present in a single organism to be resistant to nitrofurantoin. The odds of this occurring are so small that it almost never occurs (Blass 2015).

1.6 Biocompatibility:

Biocompatibility refers to the capacity of a material to act in the presence of an appropriate host response if it is applied intentionally (Schmalz and Arenholt-Bindslev 2009a). In other terms, biocompatibility reflects the potential of a material to trigger a reasonable response of the host immune system when that material is applied in the body. Biocompatibility represents the way a material interacts with its environment (Williams. 2008).

According to EN 1441 (European Committee for Standardization 1996) biocompatible materials must be free of any risk (Hauman and Love 2003a). Meanwhile, a material is considered to be biocompatible if it neither interferes, toxic, injurious, nor immune-stimulant to living tissue (Feron *et al*. 2018).

In case a material is placed in direct contact with living tissue, an interaction with the biological systems surrounding it takes place, thus resulting in a biologic response (Williams *et al*. 2008).

Materials that can function within the living tissues or in contact with biological fluids with minimal adverse reactions in the body are called biomaterials (Jenny *et al*. 2017). All biomaterials which are implemented

in the field of dentistry should be investigated for biocompatibility to protect patient health and safety (Murray *et al*. 2007).

The biocompatibility of any dental material largely depends on its release of substances because of either solubility or corrosion. Those released substances might induce destruction of the cells, or induce cellular synthesis of certain proteins leading to inflammatory response (Schmalz and Arenholt-Bindslev 2009b). Furthermore, materials can be classified into three categories depending on tissue response they induce; chemically inert, bioresorbable or bioactive (Jenny *et al*. 2017). However, a biocompatible material is not necessarily completely inert, because the type of host response is important (Wataha 2001).

1.6.1 Biocompatibility requirements of dental materials:

Some substances which may be released from dental materials, might produce biological responses locally as in the pulp, root apex, periodontium, or nearby soft tissues like buccal mucosa, tongue. These substances might enter the body systems through variable paths, thus, biocompatibility requirements include:

- They should not be harmful to the pulp, soft, or hard tissues of the oral cavity in particular and the whole body in general.
- They should not sensitize or produce allergic reactions.
- They should not undergo biodegradations.
- They should not contain any toxic diffusible substances which get released and enter into the circulatory system.
- They should not be toxic to the immune systems.
- They should not be carcinogenic.
- They should not show estrogenicity and should not contain xeno estrogens. (Schedle *et al*. 2007; Shrivastava *et al*. 2016)

1.6.2 Measuring the biocompatibility of dental materials:

Multiple factors are involved while assessing the degree of biological response. The major factors are;

- Site of the material.
- The time the material will persist inside the body.
- Stresses induced over the material.

(Shelton 2017)

Investigating the biocompatibility of any dental biomaterial involves many options as the procedures of measurement are developing and as knowledge about the interactions between dental materials and oral tissues increases and as technologies for assessment evolve (Williams 2008).

A single test is useful only for assessing one kind of undesirable reaction out of a wide range of probable reactions. For instance, tests that include cell culture can measure solely the effect of a material on isolated cells. Whereby, clinical trials in addition to animal studies, are helpful to assess the possible risks of a biomaterial to patients. The animal studies represent the nearest simulations of a material's utilization in humans (Schmalz and Arenholt-Bindslev 2009b).

In the past, any new material could be experimented in humans to find out if it was biocompatible or not. In the present time, this attitude is not acceptable any more, thus any new material should undergo thorough assessment of the biocompatibility prior to their use in humans. The assessment of the biocompatibility of any dental material involves a wide spectrum of tests due to different types of undesirable tissue reactions that might be elicited. variable tests are available to investigate the biocompatibility, these tests are classified as: in vitro, animal, and usage tests (Shelton 2017).

The biocompatibility of endodontic materials can be investigated through variable tests; the commonest tests are:

- In vitro cytotoxicity assays on cell or tissue cultures.
- Biocompatibility tests; through implantation of tested material in the subcutaneous connective tissue or bone or of experimental animals. (Soaresa *et al*. 2015)

1.6.2.1 Cytotoxicity tests:

The cytotoxicity test is regarded as a biological assessment and a screening assay, which is undertaken to measure living cell's response to an implant material in a cell culture test, involving viable cells and the capacity of cellular growth (Assad and Jackson 2019).

Although cell culture tests supply important knowledge concerning the cells' response towards a selected material, they cannot reflect the wide range of the host's tissue responses to that material if used in *vivo* circumstance. In *vivo* implantation studies will supply more complete, comprehensive, and clinically related details about the host's living tissue response (Soaresa *et al*. 2015).

1.6.2.2 Implantation tests:

Implantation tests represent a common method of in *vivo* test involving animal implantation tests. In this test, the dental material is implanted inside the animal's living tissue to investigate its undesirable reactions locally (Olsson *et al*. 1981).

Implantation tests allow proper monitoring of the diverse interactions between the animal living tissues and the tested dental material, hence, must be clinically more reliable than in vitro studies (Tayebi and Moharamzadeh 2017).

Implantation test is achieved through implanting the dental material surgically into the desired implant position in the tissue of the animal (Anderson and Schoen 2013).

Examination of the local response of the tissue due to the pathological effects of the material is performed both on two levels; gross level and microscopic level. Histological (microscopic) assessment detects different parameters of host tissue response. The animals used for short-term examination of tissue response within 12 weeks include; mice, rats, guinea pigs, or rabbits (Qin 2016).

While if longer periods (1 - 2 years) are needed to assess the biocompatibility of implanted dental materials in subcutaneous tissue, muscle, bone, or percutaneous implants in jaws, then the animals suitable for this purpose are; rats, guinea pigs, rabbits, dogs, sheep, goats, pigs, and other animals which have a longer life expectancy (Sakaguchi and Powers 2012; Raghavendra *et al*. 2015).

Several studies investigating the effects of endodontic materials utilize rodents such as Wistar rats which represent the standard model. Studies of biocompatibility involving rats are successful methods from an economic point of view for evaluating the biological effects of any dental material (Scarparo *et al*. 2009).

The rat model is applied based on the similarities with humans which was noted following the mapping of the greater region of the rat genome. Other studies may use other animals such as guinea pigs and rabbits. The procedure includes either direct implantation of the tested material or the implantation of tubes containing the tested materials, those tubes are made up of polyethylene, dentine, teflon or silicone (Kochenborger *et al*. 2009).

In biocompatibility testing involving short term evaluations, there will be an overlapping group of responses due to the physiological process of wound healing because of surgical trauma to implant the material in

addition to the immune response of host tissue to the implanted material (Anderson and Jiang 2019).

The advantage of employing tubes for implantation, with the tested dental materials inside them, is to standardize the tissue-material interaction, subsequently minimizing the need for a large amount of the tested material which result in enhancing more inflammatory response in order to simulate the clinical real status. In addition, the implantation tubes will make the retrieval of biopsies easier as the tubes will mark the site of incisions to be made, also the histological evaluation of the inflammatory response will be more precise as observing the inflammatory reaction is more accurate in well-defined zones around the implanted tubes (Kochenborger *et al*. 2009).

There are three cardinal reactions which should be examined for the evaluation of biocompatibility, including: Inflammation, wound healing, immunological reactions or immunity.

Tissue-material interactions are reactions that are elicited by both the surgical implantation technique and the presence of the tested material (Atala A *et al*. 2011).

There is controversy about which type of tests to use to evaluate the biocompatibility of intracanal medicaments, weather cytotoxic test or implantation test. Ferraz *et al*. (2001) evaluated the cytotoxic effect of chlorohexidine (CHX), they concluded that, CHX cannot be used as a routine intracanal medicament due to its cytotoxicity, in addition to the allergy that it may induce in some patients. Regarding triple antibiotics paste (TAP), Gomes-Filho *et al*. (2012) stated that TAP is a biocompatible material.

Er *et al*. (2009) studied the effects of TAP on the rat subcutaneous tissues, and they found that it stimulated a moderate inflammatory response in the first 15 days, and then declined to mild in 30 days.

Ruparel *et al*. (2012) examined the survival of the human stem cells of the apical papilla (SCAP) following contact with various dilutions of TAP, MTAP, or DAP (double antibiotic paste; metronidazole and ciprofloxacin). They stated that, 1000 mg/mL could be risky for the survival of SCAP cells during TAP dressing and consequently to the ultimate result of dentin-pulp regeneration.

Pereira *et al*. (2014) comparing calcium hydroxide with TAP, they demonstrated that TAP produced an exuberant angiogenic and inflammatory reaction, higher vascular area, and more inflammatory cells.

1.7 Effects of endodontic materials on the radicular dentin:

The main aim of endodontic treatment is get rid of microorganisms from the root canal system to prevent the periapical infections. Although failure of endodontic therapy is mostly due to persistent infection, non-microbial as well as biological factors may be contributing to endodontic failure. The mechanical properties of dentin are largely dependent on dentinal collagen. Any alteration in the crosslinks of collagen fibril will end up in brittle endodontic treated teeth which makes it friable (Soares *et al*. 2018).

The microstructure and properties of tooth dentin are essential in endodontics and restorative dentistry; hence the mechanical properties of human root canal dentin should be well understood (Bakr *et al*. 2016).

The human tooth dentin is less mineralized than the enamel being 96% of weight, while it is more than bone or cementum about 65% of weight. The dentine is about 30% organic materials which are mainly composed of collagen fibrils, whereas the 70 % is formed by inorganic material (Goldberg *et al*. 2011).

This inorganic material is constituted by calcium and phosphorus to form the hexagonal hydroxyapatite. Furthermore, small quantities of other elements like sodium, chloride, and magnesium are also present as

confirmed by the analysis done by X-ray energy dispersive spectroscopy (EDS) (Pepla *et al*. 2014).

The dentin has greater compressive, tensile, and flexural strength than the enamel, because dentine's structure is largely formed by small parallel tubules that are arranged in arrays inside collagen matrix, which is highly mineralized (Varley *et al*. 2019).

Dentinal strength is largely dependent on the cross link between hydroxyapatite and collagenous fibrils. Once dentin is exposed to disinfection agents during endodontic therapy, its physical characteristics and consequently fracture resistance were found to be affected (Elgendy and Nagy 2015).

Arends and ten Bosch (1992) and Panighi and G'Sell (1993) stated that hardness is mainly dependent on the mineral content of tooth structure, and that microhardness determination can supply indirect evidence of mineral loss or gain in dentine hard tissues as it reflects the composition and alterations in the surface of the tooth structure.

Several studies demonstrated that the use of disinfection agents (irrigation and intracanal medicaments) on root dentin during endodontic therapy may adversely affect the mechanical and physical properties such as; dentin flexure strength, hardness, resistance to fracture, and indentation properties of radicular dentin (Grigoratos *et al*. 2001; Eldeniz *et al*. 2005; De-Deus *et al*. 2006; Zhang *et al*. 2010).

Koshy *et al*. (2009) studied the effect of CH as an intracanal medicament on dentine hardness and they concluded that with long duration of contact with CH medicament, it reduces the dentin hardness and makes it less resistant to fracture.

Yassen *et al*. (2013) found that TAP caused significantly more microhardness reduction than DAP or CH medicaments, which means it caused more superficial radicular dentine demineralization.

Regarding the effect of TAP on dentinal wall thickness, Yassen and Platt (2013) concluded that application of TAP, DAP, or CH for three weeks significantly reduced the resistance to fracture than those medicaments' application for one week.

Prather *et al*. (2014) investigated the effects of TAP and MTAP concentrations on the microhardness of radicular dentin. Their result showed that TAP and MTAP significantly reduced the microhardness of roots when compared to untreated control roots, furthermore they showed that 1 mg/mL for both medicaments caused significantly less reduction in microhardness when compared to 1 g/mL.

Elgendy and Nagy (2015) showed that Propolis and TAP were adversely affects fracture resistance of root canal dentin significantly more than CHX.

Another Study by (Yassen *et al*. 2015) concluded that the use of methylcellulose-based triple antibiotic paste DTAP and CH medicaments during endodontic procedure caused significantly less microhardness reduction and superficial demineralization of dentin compared to the use of TAP.

Yilmaz *et al*. (2016) demonstrated that the application of DAP and TAP for a period of four weeks significantly reduced the microhardness values of dentin compared with the baseline values.

- **Microhardness Testing:**

Microhardness is defined as the resistance of the material to local deformation, and its tests rely on the resultant permanent surface

deformation which persists after removal of the load. Microhardness measurements can be related with other mechanical characteristics like fracture resistance, modulus of elasticity, and yield strength (Bakr *et al*. 2016).

Microhardness tests are routinely performed to evaluate the physical properties of materials, and they are largely utilized to measure the hardness of teeth. It is based on the assessment of the resistance of the dentin to a deformation induced by the penetration of an indenting stylus and is measured in units of pressure, or force per unit area of indentation (Chuenarrom *et al*. 2009; Kinney *et al*. 2003).

Multiple types of surface hardness tests are available. Many of them depend on the ability of the material's surface to resist penetration by a diamond point or a steel ball under a specified load. Those tests most commonly utilized to determine the hardness of dental materials are; Barcol, Brinell, Rockwell, Shore, Vickers and Knoop (Anusavice 2003).

Vickers microhardness test uses a square-based diamond pyramid probe to produce a diamond-shaped indentation on the surface of a material. The Vickers hardness number (HV) is calculated by an equation in which, the load is divided by the surface area of the indentation. Therefore, the larger HV, the harder the surface. To get a precise measurement, the surface of the material should be flat, mirror-like polished, and free of defects (Grote and Antonnson 2009).

The dentin microhardness test by Vickers microhardness tester is simple, fast, and requires only a tiny area of specimen surface for evaluation. During this procedure, the specimen surfaces are intended with a diamond indenter (a Vickers) at a certain load for a certain period of time. After release of the load, an optical microscope is used to measure the diagonals of the indentation (Gutierrez-Salazar and Reyes-Gasga 2003).

The hardness number is calculated by dividing the indentation load by the area of the permanent impression, which reflects the shape of indenter. The indentation load for the microhardness test can be performed using various loads from 1 to 1,000 g, and with various loading times. According to HV equation, the microhardness value should always be constant when loads are varied, because the indentation size proportionally increases according to the increase in the load and vice versa (Chuenarrom *et al*. 2009).

Vickers microhardness testing have been used in several studies to assess and quantify the effect of the tested intracanal medicament on the physical properties of radicular dentin.

Yassen *et al*. (2013) showed that application of TAP and DAP caused significant and continuous decrease in root dentine microhardness after one and 3 months respectively. The three-month intracanal application of $Ca(OH)_2$ significantly increased the microhardness of root dentine.

Prather *et al*. (2014) found that all the treatment groups showed significant reductions in microhardness of roots when compared to untreated control roots. And showed that 1 mg/mL TAP and MTAP caused significantly less reduction in microhardness when compared to 1 g/mL antibiotics.

Yassen *et al*. (2015) concluded that the use of 1 mg/mL methylcellulose-based triple antibiotic (DTAP) or Ca(OH)2 medicaments may cause significantly less microhardness reduction and superficial demineralization of dentin compared to the use of 1gm/mL TAP when applied for four weeks.

While, Yilmaz *et al*. (2016) showed that applying CH, DAP and TAP for 4 weeks reduced the microhardness values of dentin discs compared with the baseline values.

Chapter Two

MATERIALS
AND
METHODS

Materials and Methods

2.1 Materials and equipment:

2.1.1 Materials:

Item	Manufacture
Adhesive unplasticized polyvinyl chloride tape.	Graphic Tape; Chartpak, Leeds, MA, USA.
Alcohol pad	70% isoprophyl alcohol. Bolikim, China.
Alumina rotary felt disc	Akasel A-S, Roskilde, Denmark.
Brain heart infusion broth	LAB M Limited/Neogen, lancshire, UK. Exp. D: 2026
Carbide abrasive papers.	600, 1000, 1500, and 2500 grit.
198 Caries free extracted, straight single-rooted permanent human teeth.	
18 Caries free, maxillary permanent human canine.	
Centrifuge tube	CAPP, Nordhausen, Germany.
Ciprofloxacin (pure powder)	Particle size: 165-320 nm. Skywalk Pharmacy, Wauwatosa, Wisconsin, USA. Exp. 2022
Clear nail varnish.	Orly International Inc, California, USA.
Clindamycin (Pure powder)	Nanoparticle, Skywalk Pharmacy, Wauwatosa, Wisconsin, USA. Exp. 2022
Composite polishing kit.	SHOFU Dental GmbH, Ratingen, Germany.

Dental safety eyeglass	
Disposable endodontic pre-bent needle tips (20-gauge)	3D-ENSP 3D, USA.
Disposable sterile insulin syringe.	Terumo, Europe.
Disposable straight needle (23-gauge)	BD, Franklin Lakes, New Jersey, USA.
Disposable syringe (5.0 c.c.)	BD, Franklin Lakes, New Jersey, USA.
Distilled water	AstraZeneca.Boston, Massachusettes, USA.
17% EDTA. (ethylene-diaminetetraacetic acid)	Root canal preparation solution, D Line. Estonia, Europe.
Endodontic irrigating syringe.	Pacotech Inc. Texas, USA.
70% Ethanol.	Ethyl Alcohol. Mayaset, Turkey.
Filter paper discs.	United, India.
Fluid thioglycolate media.	LAB M Limited/Neogen, lancshire, UK.
Hematoxylin and eosin dye.	TissurPro, USA. Exp. 2023
Ketamine	KETA-JECT 10%, Dopharma, Netherlands. Exp. 2021
Laboratory jar	
Laboratory polypropylene rat cage	Meditech, India.
Light-cured composite resin	Tetric N-Ceram, Ivoclar Vivadent, Liechtenstein. Exp. 2021
Light-cured resin bonding agent	Tetric N-bond. Ivoclar Vivadent, Liechtenstein. Exp. 2021
Methylcellulose powder	Sigma-Aldrich. Chemie GmbH Schnelldorf, Germany. Exp. 2022

Metronidazole (pure powder)	Particle size: 163-505 nm. Skywalk Pharmacy, Wauwatosa, Wisconsin, USA. Exp. 2022
Muller Hinton broth.	LAB M Limited/Neogen, lancshire, UK. Exp. 2023
10% Neutral Formalin solution.	PolySciences, Inc, USA.
Nitrofurantoin (pure powder)	Nanoparticle. Procter & Gamble company, Cincinnati, OHIO, USA. Exp. 2023
Normal saline	
Paraffin wax.	Tianshi New Material Technology Co. LTD, Nanjing, China.
37% phosphoric acid.	N-Etch. Ivoclar Vivadent, Liechtenstein. Exp. 2021
0.9% physiological saline.	B. BRAUN Medical Inc. Pennsylvania, USA.
Polyethylene tube	DUKE, USA.
Pumice stone powder	Keystone dental, Inc, Massachusetts, USA.
Reduced transport fluid	
Self-cure acrylic resin	DLine. Estonia, Europe.
Sheep blood agar	Oxoid Limited. Hampshire, UK. Exp. 2021
Skin non-absorbable silk Suture	Pezeshk Yaran Amin Salamat company, Iran.
5.25% Sodium hypochlorite (NaOCl)	Sultan Healthcare, Pennsylvania, USA.
5% Sodium thiosulfate	The Science company, Colorado, USA.

Sterile cap	
Sterile cotton	Kardelen Yazilim, Yenisehir, Turkey.
Sterile gauze	Nantong Jianan Medical Products Co. Ltd. Jiangsu, China.
Sterile gloves	Slightly powdered natural rubber latex surgical Gloves, UNIMED, KSA.
Sterilized pack	PMS Steripack, PMS MEDIKAL, Turkey.
Sterile petri dishes	
Sterile test tube	
Surgical mask	
Temporary filling	D Line. Estonia, Europe. Exp. 2022
Tissue cassette	PolySciences, Inc, USA.
24 White albinos female Wistar rats	
Xylazine	Xyla, Interchemie, Netherlands. Exp. 2021

2.1.2 Equipment:

2.1.2.1 Instruments:

Item	Manufacture
Alumina rotary felt disc	Akasel A-S, Roskilde, Denmark.
Diamond disc double face wheel	15LC Diamond Wafering Blade, Buehler, Illinois, USA.

Endodontic barbed broach	Densply. MAILLEFER, Switzerland.
Endodontic K-file	Mani, Tochigi, Japan.
Gates glidden drills	Dentsply, Maillefer, Ballaigues, Switzerland.
Ni-Ti ProTaper rotary system, files	Densply. MAILLEFER, Switzerland.
Periodontal curette	MEDENTRA, USA.
Rubber dam	OptraDam Plus. Ivoclarvivadent, Liechtenstein.
Small amalgam stainless steel condenser	
Sterile dropper	
Sterile surgical blade	Beybi, Turkey
Sterile tweezer	MEDENTRA, USA.
Surgical forceps	MEDENTRA, USA.
Surgical handle	MEDENTRA, USA.

2.1.2.2 Devices:

Item	Manufacture
Analytical balance- Sartorius Entris 64-1S. Analytical Balance 60x0.1mg.	Sartorius Lab Insruments GmbH & Co.KG. Goettingen, Germany.
Autoclave	Zirbus Technology. GmbH, Bad Grund (Harz), Germany.

BD Phoenix system.	Becton, Dickinson and Company, Franklin Lakrs, NJ, USA.
Centrifuge device	HERMLE Labortechnik, Wehingen, Germany.
Densicheck device	DensiCHEK plus. BioMérieux. Craponne, France.
Digital Vickers microhardness tester	SCTMC, Shanghai Shangcai Tester-machine Co. Ltd. Shanghai, China.
Hair shaving machine	Ding Ling, Singapore.
Incubator	Memmert, Schwabach, Germany.
Laboratory flow cabinets	Advancelab Pte ltd, Senang Cres, Singapore.
Led light curing device	Vimel, China.
Magnetic stirring machine	Cole-Parmer Digital Magnetic Stirrer Cole-Parmer GmbH. Wertheim, Germany.
Optical light microscope	Olympus, Japan.
pH. meter device	EzDo 6011, France.
ProTaper rotary system micromotor handpiece device	NSK-NAKANISHI, Tokyo, Japan.
Stereomicroscope	Olympus, Japan.
Ultrasonic cleaner	ZZKD, Zhengzhou, China.
Vitek-II system	BioMérieux. Craponne, France.
Weigh balance	powenta, German.

2.2 Methodology:

2.2.1 Isolation of *Enterococcus faecalis:*

2.2.1.1 Patient selection:

The protocol of this study was approved by the ethical committee of the college of medicine, university of Sulaimani with no. 69.

Three isolates of *Enterococcus faecalis* were isolated from three different patients with the following criteria:

- **Patient no.1**:

The patient was admitted to hospital, complaining of high fever, rigor, tachycardia, sweating, leukocytosis, elevated ESR (Erythrocyte Sedimentation Rate) and CRP (Compliment Reactive Protein). The patient was diagnosed with sepsis and *E. faecalis* was retrieved in the result of blood culture and this isolate of *E. faecalis* was labeled as isolate one (S1).

- **Patient no.2**:

The patient was diagnosed with failed endodontic treatment without local or systemic antibiotic administration within the last three months and the patient was in need for retreatment. That isolate of *E. faecalis* which was isolated from the root canal system in this patient was labeled as isolate two (S2).

The criteria for diagnosis of failed endodontic treatment according to Vineet *et al*. (2016) included; defective coronal seal with incomplete obturation of the root canal that was short filling more than 2 mm shorter than the radiographic root apex, (Radiographic presence of voids and radiolucent space running along some of the working length of root filling) with a periapical radiolucency demonstrated in the periapical radiograph. Additionally, the tooth could be isolated with a rubber dam and with no periodontal pockets more than 4 mm.

The exclusion criteria for selecting the patient were; smoking, pregnancy, diabetes mellitus, autoimmune disease, chemotherapy, immunosuppressive therapy, and malignancy.

- **Patient no.3**:

This patient was also diagnosed with failed endodontic treatment but was on a course of antibiotics for the last two weeks with no response and the patient was in need for retreatment. This isolate was labeled as isolate three (S3).

The criteria for the diagnosis and the exclusion criteria were the same as outlined in patient no. 2.

2.2.1.2 Sampling protocol:

The patients were interviewed, informed thoroughly about the study purpose, and informed written consent was signed before taking the sample. After obtaining a previous dental history, the patient's chief complaint was documented, and a clinical examination was performed and correlated with radiographic findings.

2.2.1.3 Sampling procedure:

In patient no.1, blood samples were taken and sent for blood culturing on blood agar in the bacteriology department (Central Laboratory, teaching hospital, Sulaimani), and incubated at 37°C for 48 hours and was examined by a bacteriologist and documented to be *E. faecalis*, this was considered as (S1).

The second and third isolates (S2) and (S3) isolated according to the procedure of root canal swabbing described by (Gomes *et al*. 2004) and (Vineet *et al*. 2016).

The selected tooth was cleaned with pumice stone powder and isolated with a rubber dam then it was disinfected with 5.25% sodium hypochlorite

(NaOCl) for 30 s, after that it was inactivated with 5% sodium thiosulfate. The whole technique was under aseptic conditions. After removing tooth filling, the root canal orifice was identified, followed by sterilization of the pulp chamber with 5.25% sodium hypochlorite, previous obturation was removed with Gates Glidden drills and endodontic files. Sterile saline was introduced inside the canal lumen to wet the canal. Then two sterile paper points were inserted into the full length of the canal and kept for 60 s. The paper points were placed into a 3-mL centrifuge tube containing 3 mL of reduced transport fluid (RTF) and transported to the microbiology department to perform the microbiological processing.

2.2.1.4 Laboratory confirmation of *Enterococcus faecalis* species:

Three different isolates were confirmed by the Phoenix and Vitek-II system and by 99% identification with automated sensitivity reporting for all isolates. The *E. faecalis* isolates were cultured in brain heart infusion (BHI) broth and stored at 37°C for 48 h. The three isolates of *E. faecalis* subcultured in blood agar is shown in (Figure 2.1)

Figure 2.1 The isolated three isolates of *Enterococcus faecalis.*

2.2.2 Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of nitrofurantoin and modified triple antibiotic mixture against *Enterococcus faecalis*.

2.2.2.1 Preparation of bacterial suspension:

To achieve a bacterial suspension with a concentration of 0.5 McFarland containing 1.5×10^8 cells/mL, the microbial cells were resuspended with saline. Densicheck device was used to measure the optical density of the microorganism suspension, as shown in (Figure 2.2).

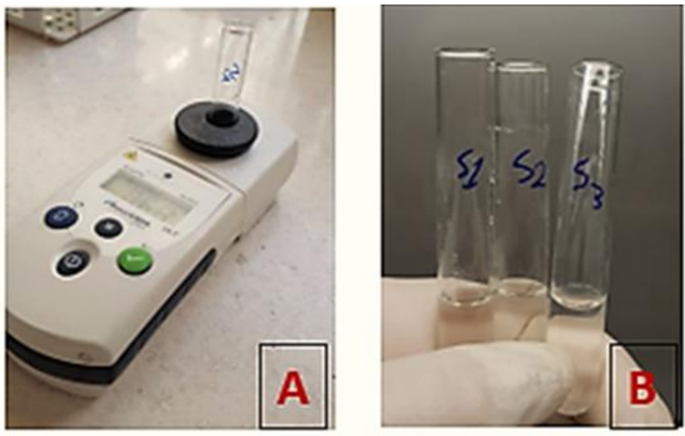


Figure 2.2 Preparation of the bacterial suspension **A.** Densicheck device **B**. Suspensions of the three isolates of *Enterococcus faecalis* (S1, S2, and S3).

2.2.2.2 Serial dilution method:

In this study we used a pure powder of each antibacterial agent: (nitrofurantoin) and (ciprofloxacin, metronidazole and clindamycin) to prepare triple antibacterial mixture. A sensitive weight measure for calculating the weight of the antibiotic powder was used. For the preparation of the antibiotic solution**,** the following procedure was similar for both groups:

- **Group (Nitrofurantoin)**:

One hundred mg of nitrofurantoin pure powder was dissolved in 0.5 mL of distilled water. Distilled water was added until the solution reached the final volume of 1 cc to obtain a primary concentration of 100 mg/mL of nitrofurantoin solution. Next, 0.5 ml of the distilled water was poured into seven sterile tubes. Then, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and 1/128 concentrations of the primary solution of nitrofurantoin were prepared.

- **Group (Triple antibacterial mixture)**:

A pure powder mixture of 100 mg of ciprofloxacin, 100 mg of clindamycin, and 100 mg of metronidazole were dissolved in 0.5 ml of distilled water. Distilled water was added until the solution had reached the final volume of 1 mL to obtain a primary concentration of 100 mg/ml of triple antibacterial solution. Then 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 concentration of the primary solution of triple antibacterial mixture were prepared. As a result, the following concentrations were obtained for both nitrofurantoin and triple antibacterial solutions: 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.12 mg/mL, 1.56 mg/mL, and 0.78 mg/mL. The procedure above repeated three times for each group to prepare tubes for the three isolates of *E. faecalis*.

2.2.2.3 Minimal inhibitory concentration (MIC) evaluation:

Preparation of the inoculum was achieved by making a direct broth suspension of isolated colonies of *E. faecalis* selected from the 24 hour blood agar plate , using the bacterial suspension of 0.5 McFarland turbidity standard. Then dilution of the adjusted inoculum suspension in Muller Hinton broth was done so, after inoculation, each tube contains approximately 1.5 x 10^5 CFU/mL. 1 mL of the adjusted inoculum is added to each tube containing 1 mL of antimicrobial agent in the dilution series

and mixed. Then Incubation of the inoculated tubes at 37 ºC for 24 hours in an incubator. The MIC was determined by visual examination for turbidity. The lowest concentration at which the isolate is completely inhibited (as evidenced by the absence of visible bacterial growth) is recorded as the minimal inhibitory concentration or MIC.

The MIC results for nitrofurantoin were compared with the CLSI (clinical and laboratory standards institute) being:

Sensitive (S) if the MIC is ≤ 32 mg/mL, Intermediate (I) if the MIC = 64 mg/mL, and Resistant (R) if it is ≥ 128 mg/mL. (CLSI's Performance Standards for Antimicrobial Susceptibility Testing. 2019).

2.2.2.4 Minimal bactericidal concentration (MBC) evaluation:

To determine the MBC, a loopful of broth from the dilution representing the MIC and two of the more concentrated dilutions were inoculated into blood agar plate and after incubation for 24 hours, were enumerated to determine viable CFU/mL. The MBC is the lowest concentration that demonstrates a pre-determined reduction (such as 99.9%) in CFU/ml when compared to the MIC dilution.

2.2.3 Assessment of the antimicrobial efficacy of nitrofurantoin and modified triple antibiotic mixture against *Enterococcus faecalis,* using zone of inhibition (a Kirby-Bauer test), (Agar diffusion method):

2.2.3.1 Preparation of test microorganism:

Enterococcus faecalis isolates were stocked and subcultured on sheep blood agar to have fresh growth inoculum. The inoculum for the antimicrobial activity was prepared by using McFarland reader to make a bacterial suspension in normal saline, with turbidity equal to 0.5 McFarland standards (Shah *et al*. 2014).

2.2.3.2 Preparation of antibiotic suspension:

Nitrofurantoin and modified triple antibiotics mixture were prepared by mixing pure powder of the antibiotics with distilled water to have seven concentrations (100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.12 mg/mL, and 1.56 mg/mL) which were applied on blood agar inoculated with standard McFarland bacterial sample.

2.2.3.3 Preparation of the antibiotics disc and wells:

For those concentrations less than 25 mg/mL, the antibiotic was impregnated on filter paper discs (about 6 mm in diameter) (agar-disk diffusion method) (Balouiri *et al*. 2016).

Meanwhile, for concentrations equal to or higher than 25 mg/mL, a well was created in the agar by a hole with a diameter of 6 mm punched aseptically with a sterile cork borer or a tip. Then, a volume (100 μL) of the antimicrobial agent was used to have the same diameter and depth to add the same amount of the antibiotic (agar-well diffusion method). (Valgas *et al*. 2007).

2.2.3.4 Screening of antibacterial activity (Agar diffusion method):

A total of 36 Petri dishes were prepared and divided into two main groups; group N; nitrofurantoin (n=18), and group M; modified triple antibiotic mixture (n=18). Each group was subdivided into three subgroups (n=6) according to the isolate of *E. faecalis;* (S1, S2, and S3), as illustrated in (Figure 2.3).

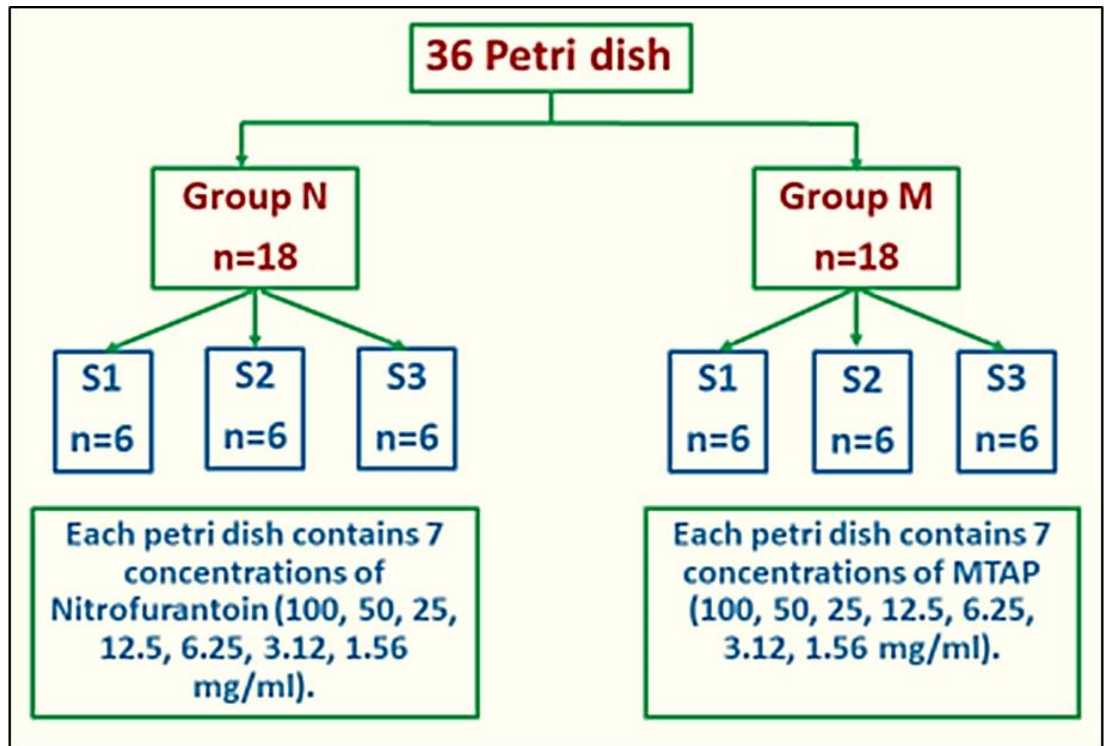


Figure 2.3 A diagram showing the distribution of the petri dish among the groups.

Each petri dish contained seven concentrations of the assigned antibiotic (100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.12 mg/mL, and 1.56 mg/mL), for each *E. faecalis* isolate (S1, S2, and S3). A lawn culture of the test organisms from the bacterial suspension was made, matching 0.5 McFarland's standard. The plates were incubated in an incubator at 37°C overnight, and the zone of inhibition of growth around the antibiotics (disc or well) was measured in millimeters. The largest diameter was recorded (Dianat *et al*. 2015).

The results obtained were correlated with the standard of (CLSI) which indicate:

Sensitive (S) when the zone of inhibition is ≥ 17mm, Intermediate (I) = 15-16mm, while it is Resistant (R) if the zone of inhibition ≤ 14 mm.

2.2.4 Assessment of the antimicrobial efficacy of nitrofurantoin and MTAP against *Enterococcus faecalis* (in ex-vivo):

2.2.4.1 Tooth preparation:

A total of 198 caries-free, straight single-rooted extracted human teeth were collected and stored in 0.9% physiological saline at room temperature until the time of use (George *et al*. 2017). The crowns were cut perpendicularly to the long axis of the teeth from the cementoenamel junction (CEJ), with a rotary double cutting faced diamond disc in conjunction with physiological saline irrigation, and kept in 0.9% physiological saline.

The root length was cut and standardized to 15 mm. After removing pulp tissue, canals were evaluated for apical patency and checked to have only one canal using #15 K-file (roots with two canals were excluded from the study. The working length was determined by one mm short of the root apex, using size 15 K-file, getting 14 mm working length. (By pushing the file until it emerge from the apical foramen then subtract 1mm).

The coronal third of the root canal was flared using Gates-Glidden drills (#1, 2 and 3) (Sousa *et al*. 2015), and the canals were instrumented within the working length with the Ni-Ti ProTaper rotary system (using sizes of S1, S2, F1, F2 and F3) at 300 rpm (rotation per minute) speed and 2.5 Ncm (Newton centimeter) torque with a micro motor handpiece. Using S1 to prepare the coronal $1/3^{rd}$ of the working length, then S2 was used until the middle $1/3^{rd}$, after that using F1, followed by F2 and F3 for full working length preparation (Unal *et al*. 2012).

After each instrument change, 5 ml of 5.25% NaOCl was used for irrigation. Then, the samples were irrigated with 5 ml of 17% EDTA (ethylene- diaminetetraacetic acid) for smear layer removal. In order to achieve the effects of EDTA, a flush with 5.25% NaOCl for 5 min was

done by using a special endodontic irrigating syringe. Then, each root was rinsed with 10 mL of physiological saline to remove the remnants of EDTA and NaOCl (Grande *et al*. 2006), then the canal was dried by using sterile absorbable paper point. Finally, the apical foramen of the root was sealed with a bonding agent and light-cured composite resin to prevent bacterial leakage. To prevent bacterial leakage from the accessory lateral canals, three layers of clear nail varnish were placed over all external root surfaces except for coronal access and with care not to occlude the root canal entrance and teeth were allowed to dry (Chockattu *et al*. 2018).

2.2.4.2 Sterilization of specimens:

Each root specimen was placed in a sterile test tube containing brain heart infusion (BHI) broth, the tubes were placed in a large laboratory jar, as shown in (Figure 2.4), and autoclaved twice for 30 min at a temperature of 121°C and a pressure of 15 PSI (Ooi *et al*. 2019). Then, those samples were kept in an incubator at 37° C for 24 hours. Bacterial viability (contamination) and broth purity were checked.

Figure 2.4 Sterilization of specimens. **A**. Each root specimen was placed in a sterile test tube containing brain heart infusion broth. **B**. Putting the test tubes in large laboratory jar for sterilization.

2.2.4.3 Inoculation of *Enterococcus faecalis* bacteria into the specimens:

All the steps of the bacteriology workup were done in a microbiological safety cabinet (hood). All the samples (roots) were taken out of the (BHI) broth test tube aseptically by using a sterile tweezer. Then the specimen was held by a sterile alcohol pad to prevent contamination of the outer surface of the sample. The broth remaining inside the root canal was removed by aspiration using a sterile disposable syringe with a small gauge needle. The canals were inoculated with the suspensions of the three different isolates of *E. faecalis* (S1, S2, and S3), with a standard concentration of 0.5 McFarland (1.5×10^8 CFU/ml), using Densicheck devise to measure the optical density for each isolate of *E. faecalis* suspension.

Then the sterilized canals were filled with 20 µL inoculums of bacteria according to the isolates by using a syringe with a sterile endodontic needle without spillage. Then the orifice of the canal was closed by a sterile small cotton pellet and sealed with eugenol free readymade temporary filling.

The specimen (root) was wrapped by wet sterile gauze and was inserted in a new sterile test tube, and the cap was closed. The tubes were put in a sterile large laboratory jar and incubated for 21 days at 37° C (Tagelsir *et al*. 2016).

2.2.4.4 Samples grouping:

The flowchart of the sample division is described in (Figure 2.5). One hundred ninety-eight roots were divided blindly into three main groups:

- Group N (Nit) (n=90) subdivided into three subgroups (n=30), according to the isolate of *E. faecalis* (S1, S2, S3) then each subgroup was divided into 5 groups (n=6) according to the concentrations used in

MIC test of nitrofurantoin used (6.25 mg/mL, 12.5 mg/mL, 25 mg/mL, 50 mg/mL, and100 mg/mL).

- Group M (MTAP) (n=90) subdivided into three subgroups (n=30), according to the isolate of *E. faecalis* (S1, S2, S3), then each subgroup was divided into 5 groups (n=6) according to the concentrations used in MIC test of MTAP used (6.25 mg/mL, 12.5 mg/mL, 25 mg/mL, 50 mg/mL, and100 mg/mL).
- Group W (n=18) (using distilled water as a negative control). Then each group was subdivided into three subgroups (n=6) according to the isolate of *E. faecalis* used (S1, S2, and S3).

Bacterial viability was checked in three randomly selected tubes for each subgroup.

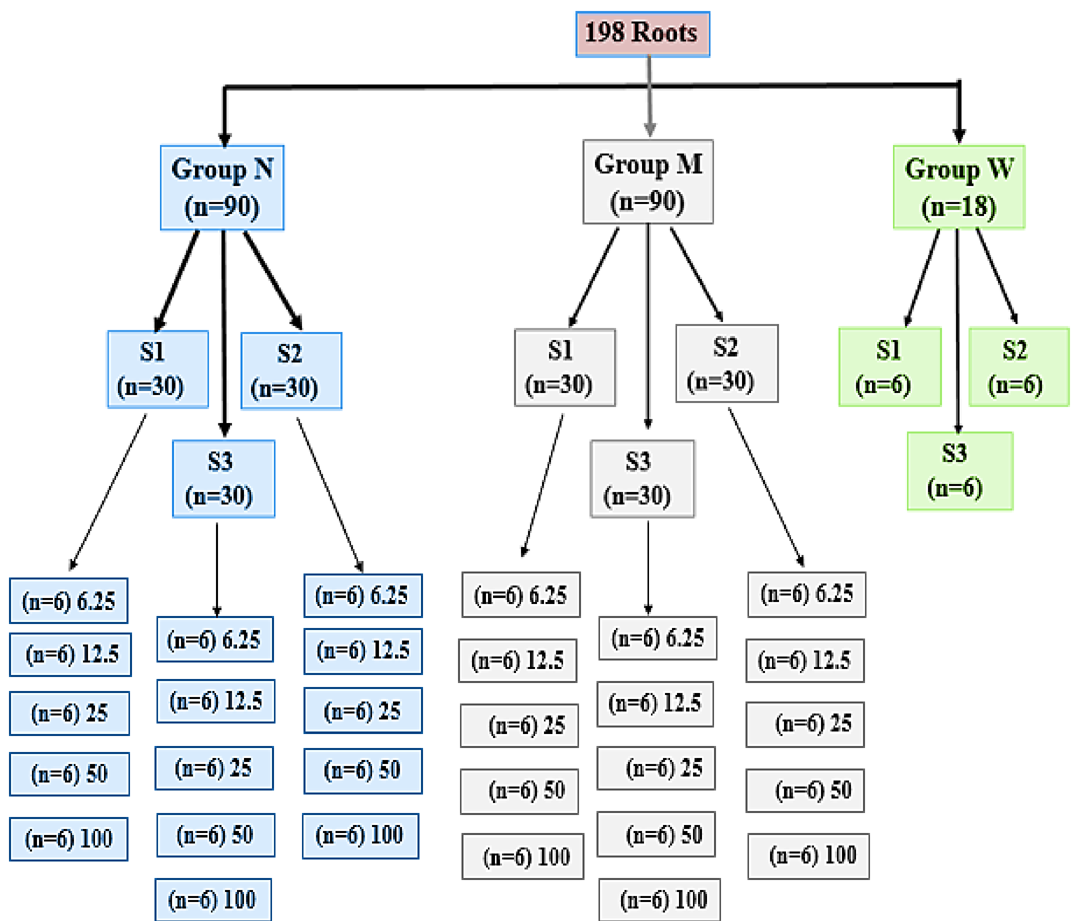


Figure 2.5 Flowchart showing the distribution of the roots among the groups.

2.2.4.5 Preparation of intracanal medicaments:

Four pure antibacterial powders were used; nitrofurantoin (Nit), and (ciprofloxacin, metronidazole, and clindamycin), for MTAP. To calculate the required amount of the antibacterial powder, an analytical balance was used. In this study, we prepared five concentrations of each medicament paste (Nit, MTAP), the concentrations are (6.25 mg/mL, 12.5 mg/mL, 25 mg/mL, 50 mg/mL, and100 mg/mL). To obtain a homogenous antibacterial paste, a magnetic stirring was used for 2 hours at room temperature.

- Group N (Nit paste): nitrofurantoin solution was prepared by mixing pure powder of nitrofurantoin with distilled water. Methylcellulose powder (MC) was added to the nitrofurantoin solution to get a thick paste-like consistency mixture (Alyas *et al*. 2016).

100 mg (nitrofurantoin) + 1 mL (distilled water) + 80 mg (MC) to prepare 100 mg/mL Nit paste. While to prepare 50 mg/ml Nit paste; 50 mg (nitrofurantoin) + 1 mL (distilled water) + 80 mg (MC) were mixed, and so on, for the other concentrations.

- Group M (MTAP): to prepare the modified triple antibiotic solution, equal proportions of pure powder of; metronidazole, ciprofloxacin, and clindamycin were mixed with distilled. To prepare MTAP (modified triple antibiotic paste), Methylcellulose powder was added to modified triple antibiotic solution to get a thick, paste-like consistency.

To prepare 100 mg/mL MTAP paste, 100 mg (metronidazole) + 100 mg (ciprofloxacin) + 100 mg (clindamycin) + 1 mL (distilled water) + 80 mg (MC) were mixed. While, to prepare 50 mg/mL MTAP; 50mg (metronidazole) +50 mg (ciprofloxacin) + 50 mg (clindamycin) +1 mL (distilled water) + 80 mg (MC) were mixed, and so on for the other concentrations.

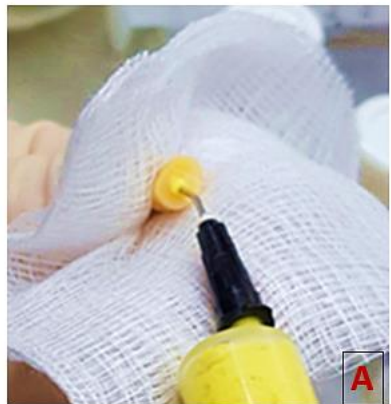
- Group W: 80 mg of (MC) was added to 1 mL of distilled water, to form MC paste.

2.2.4.6 Application of the medicament:

After 21 days of incubation, the contaminated roots were taken out of the incubator. Each root was removed from the test tube, and the gauze was unwrapped. The specimen was cleaned with an alcohol pad. The temporary filling and the cotton pellet were removed, and the canal content was aspirated. The aspirated content was cultured on blood agar for evaluation of bacterial viability and measurement of colony forming unit (CFU). Then, the root canal was irrigated with 5 ml distilled water to remove the bacterial suspension, and the canal was dried using three paper points. The nitrofurantoin and MTAP paste were prepared, as mentioned before.

Each prepared medicament was injected into root canals by using size 20-gauge pre-bent needle tip (adjasted to a disposable syringe; 5 cc) , until the canal was filled with the medicament paste, as shown in (Figures 2.6).

The roots of the negative control group were injected with MC paste in the same way the medicament was injected. A sterile cotton pellet covered the canal orifice and sealed with temporary filling, and the root was wrapped again with sterile wet gauze and placed inside a new sterile test tube (Figures 2.7). The specimens were returned to the incubator and kept there for seven days at 37°C.



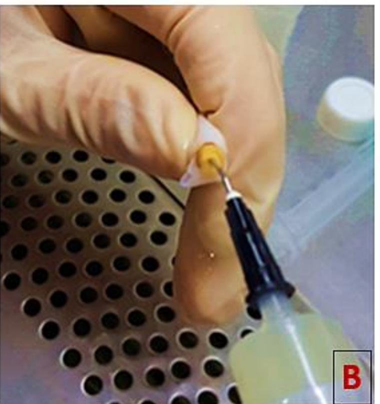


Figure 2.6 Application of the intracanal medicament. **A.** Application of nitrofurantoin paste to the specimen. **B.** Application of modified triple antibiotic paste (MTAP) to the specimen.

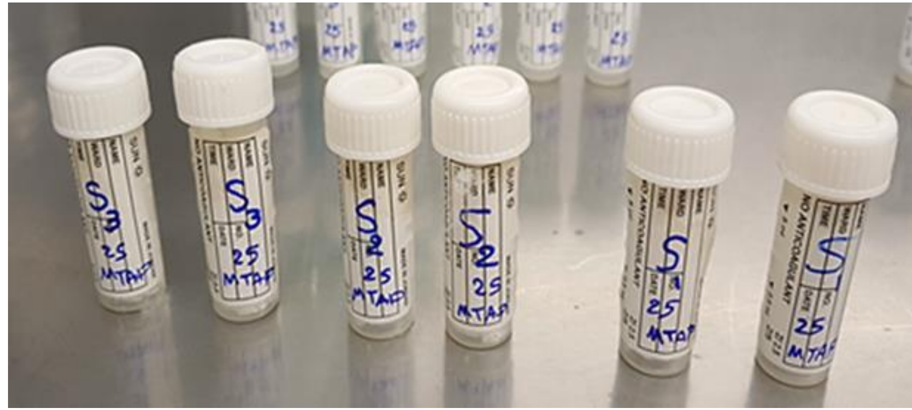


Figure 2.7 One of the subgroups: (25mg/mL MTAP)

2.2.4.7 Sampling of the canal lumen content:

At the end of the seven days of incubation, the specimens were removed from the test tube, the gauze was unwrapped, the temporary filling and the cotton pellet was removed, and the specimen was held in a sterile alcohol pad.

Intracanal medicaments were evacuated from canals by irrigation with 10 mL of distilled water by using a sterile syringe. Then two paper points were inserted into the canals were and kept for 60 seconds (Chinni *et al*. 2011). Then, those paper points were kept into thioglycolate broth through sterile test tubes. They were then incubated at 37°C for 24 hours. Then subculturing is performed on blood agar at 37 °C for 48 h. Growing colonies were counted and recorded as colony forming units CFU. To count the colonies of bacteria, we used the classical counting technique in the colony counter, and the results were given as a number of CFU.

2.2.4.8 Sampling of the dentinal chips:

After the above step, assessment of the extent of infection in the radicular dentin was done depending on dentinal chips, which were obtained by shaving the full length of the root canal using a sterile # 40 k-file. (Tip diameter 0.40 mm) (Metzger *et al*. 2010; Attia *et al*. 2015).

The dentinal chips were transferred by placing the file (just its cutting surface) into thioglycolate broth through sterile test tubes for 60 seconds (Figure 2.8), and incubated at 37°C for 24 hours. Then subculturing is performed on blood agar at 37 °C for 48 h. Growing colonies were counted and recorded as CFU.

Figure 2.8 Incubation of dentine chips harvested from the samples.

2.2.5 Evaluation of the biocompatibility of nitrofurantoin paste and MTAP in rats' subcutaneous connective tissue:

2.2.5.1 Animals and experimental proceedings:

This study was performed in accordance with the principles and guidelines of the Institutional Animal Care and Use Committee during the study (Suckow and Lamberti 2017). Ethical clearance was obtained for the undertaken study, from the college of medicine, university of Sulaimani with no. 69. All the procedures have been done in the animal house, college of veterinary, university of Sulaimani.

Twenty-four healthy white albino female Wistar rats (age 6 months) weighing 180–200 gm were used. The animals were kept in polypropylene cages marked according to the group (study period). The animals were subjected to a light/dark cycle of 12 h, mean temperature of 22°C, 40-60% humidity, with food and water provided ad libitum rations except for the preoperative 12 hours.

2.2.5.2 Groups division:

The rats were distributed randomly into four groups (n=6 rats) according to the experimental duration; three, seven, fourteen, and thirty days.

2.2.5.3 Surgical procedure:

All surgical procedures performed on the rats were done under general anesthesia by intramuscular administration of 0.1 mL of ketamine hydrochloride associated with 0.05 mL of xylazine hydrochloride per 100 g of rat's body weight. Anesthesia was delivered with a disposable insulin syringe. Then the dorsal side of the animal was shaved and cleaned with 70% ethyl alcohol.

2.2.5.4 Preparation of polyethylene tubes with the test medicaments:

Ninety-six sterile polyethylene cylinder tubes with 1.3 mm internal diameter and 10 mm length were selected: The cylinder tubes were fully filled with the tested medicament paste using endodontic pre-bent needle tips (adjusted to a sterile disposable syringe), and small condensers for material condensation.

The polyethylene tubes were divided into four groups (n=24) as following:

- Tested group 1 (TG1): The tubes were filled with the experimental paste (nitrofurantoin) at a concentration of 12.5 mg/mL. This concentration was prepared by mixing 12.5 mg of pure powder of nitrofurantoin, with 1 mL of distilled water to form nitrofurantoin solution. Then 80 mg of methylcellulose powder (MC) was added to the nitrofurantoin solution to get a thick paste-like consistency mixture.
- Tested group 2 (TG2): The tubes were filled with the experimental paste at a concentration of 25 mg/mL. This paste was prepared by mixing 25 mg of pure powder of nitrofurantoin with 1 mL of distilled water and adding 80 mg of MC.

- Positive control group (PC): the tubes were filled with MTAP at a concentration of 25 mg/mL. This concentration was prepared by mixing equal proportions of pure powder of the antibiotics; 25 mg of metronidazole, 25 mg of ciprofloxacin, and 25 mg of clindamycin, with 1 mL distilled water to prepare the modified triple antibiotic solution. To get a thick paste-like consistency mixture, 80 mg of (MC) was added to this solution to obtain MTAP. A sensitive weight measure was used to calculate the required amount of the antibiotic powder.
- Negative control group (NC): The tubes were left empty.

2.2.5.5 Inoculation of the test medicament:

Four longitudinal incisions (12 mm length each) were made in the dorsal region; two in the upper and two in the lower regions of the dorsal aspect (4 cm apart horizontally and 6 cm apart vertically), using a #15 scalpel blade. The cutaneous tissue was pinched lateral to the incisions, and the tissue was dissected using blunt-end dissecting scissors to create a subcutaneous space for the polyethylene tubes. Then the tubes were implanted into the subcutaneous connective tissue according to the groups and sites. The distribution of the groups was standardized for all the rats as the following: TG1 in the upper left incision, TG2 in the upper right incision, NC in the lower left incision and PC in the lower right incision), as illustrated in (Figure 2.9). After implantation, the skin was closed with 04 silk suture, three stiches for each incision (Figure 2.10), and left for periods of 3, 7, 14 and 30 days.

During those periods the rats were kept in their cages. After 3, 7, 14, and 30 days, six animals were euthanized by anesthetic overdose. the dorsal side of the rats was shaved and the sutures were removed. Subsequently, the tissues containing the tubes were excised and kept in tissue cassettes (Figure 2.11).

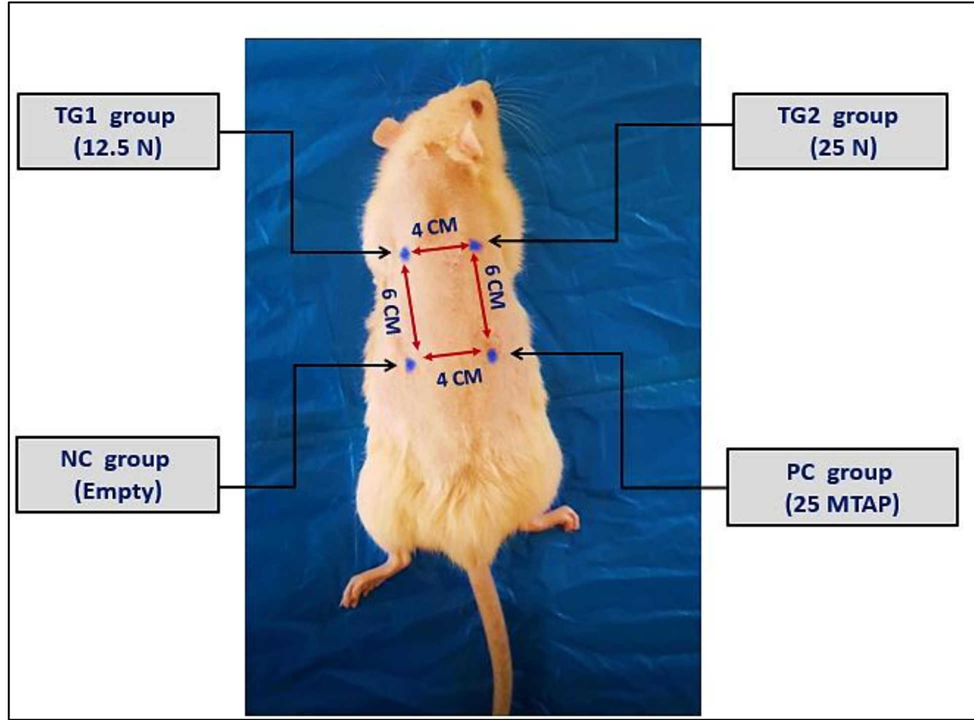


Figure 2.9 The sides of the incision and implantation of the tubes on the dorsal side of the rat.

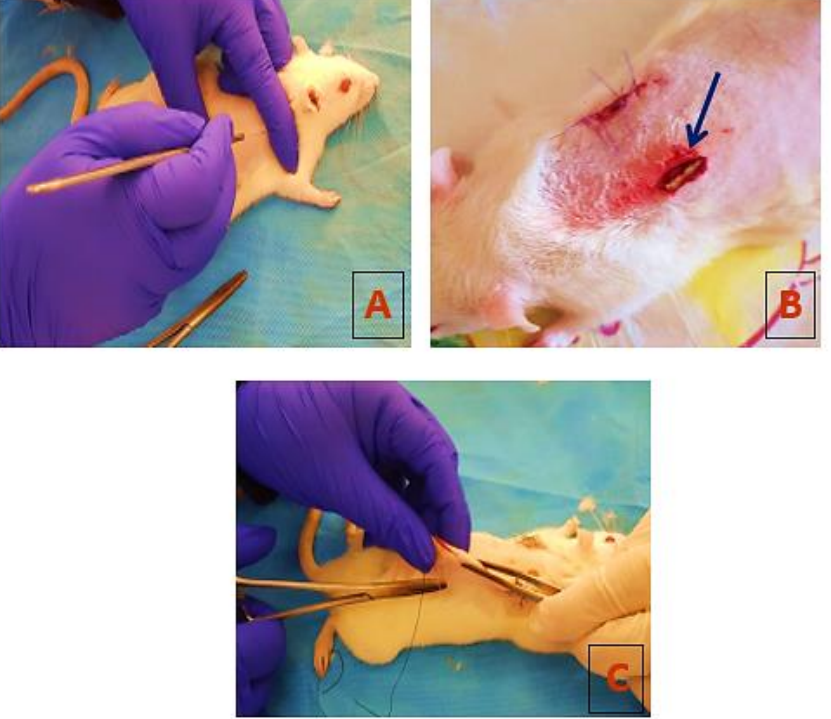


Figure 2.10 The surgical procedure for the tubes implantation. **A**. Longitudinal incisions were made in the dorsal using a #15 scalpel blade. **B**. The polyethylene tubes were implanted into the subcutaneous connective tissue. **C**. The skin incision was sutured with 04 silk after implantation.

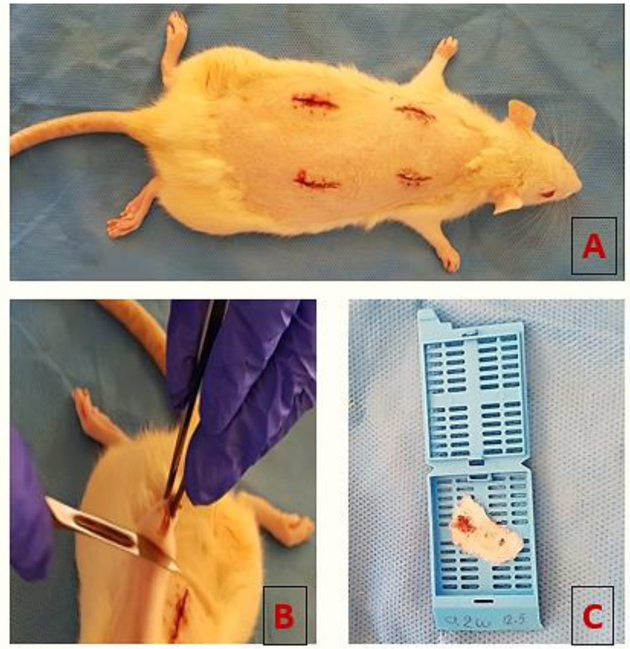


Figure 2.11 Resection of the subcutaneous tissue containing the tubes. **A.** One of the rat which euthanized by anesthetic overdose and the dorsal side of the rats was shaved and the sutures were removed. **B.** The tissues containing the tubes were excised. **C.** Putting the specimen in the tissue cassette.

2.2.5.6 Histological procedures and analysis:

The tissue cassette containing the specimens with the implanted polyethylene tubes was fixed in 10% neutral formalin for 48 hours. After that, the specimens were dehydrated and embedded in paraffin wax. Longitudinal sections 5-µm thick were obtained at every 50 µm parallel to the tube longitudinal axis, adding up to 12 sections per specimen. Hematoxylin and eosin dye was used to stain the sections. The histopathological evaluation of specimens was done with an optical light microscope (under a magnification of x100), and was performed by a pathologist who was blinded to the tested substances. The histological sections were examined for the presence and type of inflammatory process as well as the proliferation of connective tissue and presence of tissue necrosis or abscess. The degree of inflammatory process was classified

following established scores that varied according to the intensity of the inflammatory process:

The occurrence of inflammatory responses was scored according to the established scores; 0 (no reaction) for absence of inflammatory cells; 1 (mild reaction) for presence of mild chronic inflammatory infiltrate, or few eosinophilic or giant cells; 2 (moderate reaction) for presence of moderate chronic inflammatory infiltrate, or some eosinophilic or giant cells, or 3 (severe reaction) for presence of an intense chronic inflammatory infiltrate, large number of eosinophilic or giant cells (Mousavinasab *et al*. 2012).

2.2.5.7 pH measurement:

It is challenging to measure the pH of a paste because it is difficult to get an accurate value. Therefore, a saturated solution was used to measure the pH of the 12.5 mg/mL, 25 mg/mL of nitrofurantoin and 25mg/mL MTAP. This solution was made with sterile water in triplicate and measured using a digital pH meter. The meter was calibrated using pH standards of 4 and 7, as shown in (Figure 2.12). Solutions tested were placed in direct contact with the electrode and left in place for at least 15 seconds or until a stable pH reading was acquired. (maximum of 30 seconds). The electrode was rinsed with de-ionized water and wiped dry between readings.

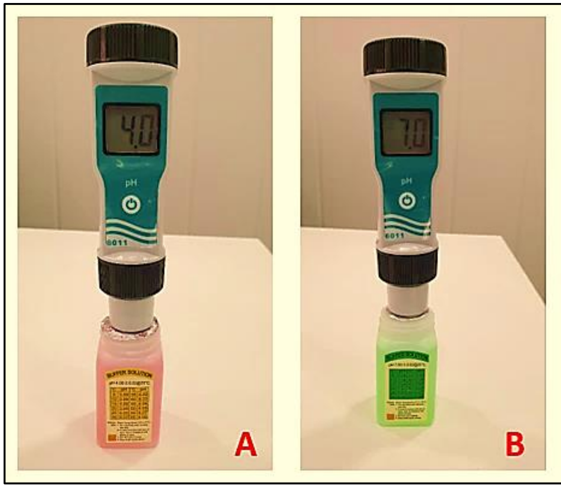


Figure 2.12 A digital pH meter was calibrated using pH standards of 4 and 7.

2.2.6 Evaluation the microhardness of the radicular dentin after using the experimental intracanal medicaments:

In this study a Vickers microhardness tester (Figure 2.13) was used to evaluate the effect of Nit paste and MTAP on the radicular dentin.

Figure 2.13 Digital Vicker microhardness tester

2.2.6.1 Specimens preparation:

A total of eighteen extracted human maxillary canine teeth with the following criteria: roots were completely formed, without calcifications or resorptions internally, and no exaggerated root curvatures. They were extracted for periodontal reasons. The canine teeth selected were from patients aged 40 to 50 years. Selection of those teeth was according to morphological and dimensional similarity.

Periodontal curette was used to remove soft tissue remnants, debris, and calculus, on the root surfaces. Storage of the teeth was in normal saline at room temperature, radiographical confirmation of a patent single canal is done in proximal view. The crowns were cut at the cementoenamel junction perpendicularly to the long axis of the teeth with a rotary double faced diamond disc under continuous water cooling. The root length was cut and

standardized to 15 mm. Biomechanical preparation was performed for all the canals in the same way as mentioned in previous experimental ex-vivo test. As a final step, each root was rinsed with 10 mL of physiological saline to remove the remnants of EDTA and NaOCl.

Three cylinders (each 5mm in length) were obtained by cutting the root horizontally to form the following parts; (C=coronal third, M=middle third, and A=apical third). The instrumented three parts (thirds) of the root were embedded in autopolymerizing acrylic resin block, as shown in (Figure 2.14).

Each block received the three thirds of the same root. The coronal aspect of each third was placed with the same level of the acrylic block surface for convenient accessibility of the root canal. Plastic rings were used as molds to form similar diameter blocks of the freshly prepared auto polymerized resin which was poured into those rings. After curing of the resin, the excess material which was present on the tooth surface was removed by polishing of specimens’ surface.

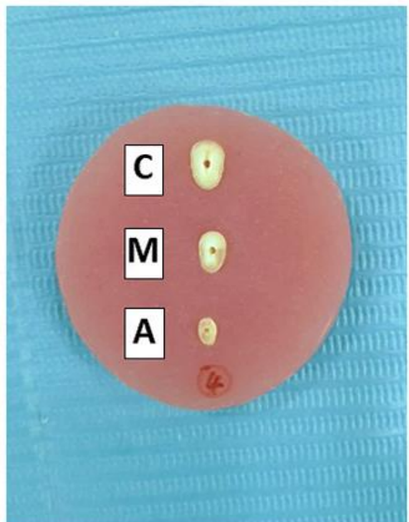


Figure 2.14 Three root thirds; (coronal, middle and apical third) were embedded in auto polymerizing acrylic resin block.

A process of grounding and smoothening of the surface of root dentin was achieved with a series of ascending grades of carbide abrasive papers (600, 1000, 1500, and 2500 grit) with cooling by distilled water to get rid of any unwanted surface scratches. Eventually, polishing was performed using fine grades of composite polishing kit, then to obtain a smooth glossy mirror-like surface, 0.1 mm alumina suspension on a rotary felt disc was used. A stereomicroscope was used to confirm that all specimens were cracks-free with no surface defects.

2.2.6.2 Microhardness measurement:

All sample blocks underwent ultrasonic cleansing in a cycle of 3 min. Then they were placed on the stage of Vickers microhardness tester, where marking of indentations were done using a Vickers diamond indenter with a load of 50 gm for a duration of 10 seconds under a magnification of x100 (Figure 2.15).

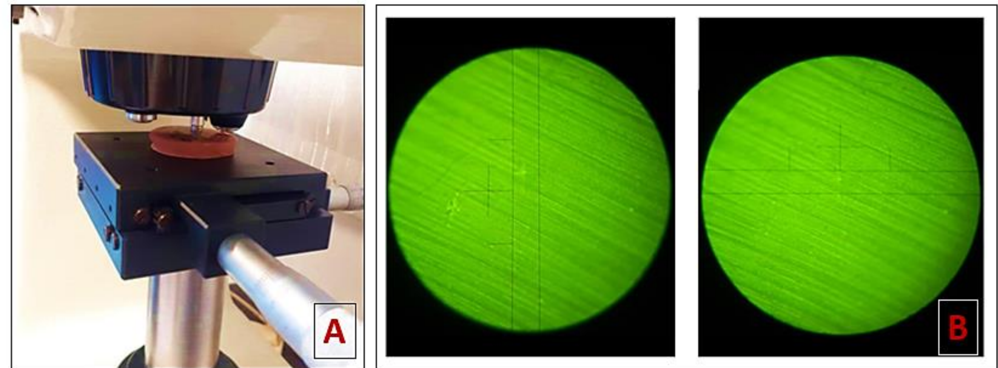


Figure 2.15 Microhardness testing of dentin samples. **A.** Mounting the sample block on the stage of Vickers microhardness tester. **B**. Microscopic view of indentation and measurement of microhardness value (HV).

2.2.6.2.1 Pre-treatment microhardness measurement:

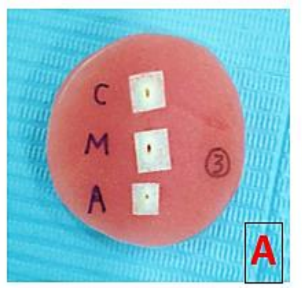
Before application of the tested pastes, measurement of the microhardness values (HV) was performed on the polished coronal surface of each root third as a baseline value and considered as (pre-treatment)

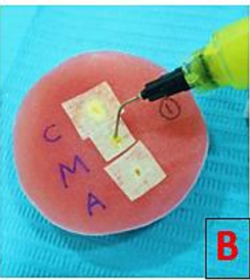
measurement. Two indentations were done on the mesial and distal sides of each specimen third, as shown in (Figure 2.18). The landmark for indentation was standardized at 500µm from the canal wall (Soram *et al*. 2016). Microhardness values were recorded as the mean of the two indentations measures.

2.2.6.2.2 Samples grouping:

The resin blocks (containing the three root thirds) were randomly divided into three groups; 25 mg/mL nitrofurantoin (n = 6 blocks), 25 mg/mL MTAP (n = 6 blocks), and control group (no medicament) (n = 6 blocks).

For the control group, sterile distilled water was applied into the canal. For the treatment groups, the exposed dentin was covered with an adhesive unplasticized polyvinyl chloride leaving only the canal lumen exposed. The intracanal medicament paste was inserted into the canal lumen using a disposable syringe with an endodontic flat tipped angled needle, then the canal orifice was covered by the tape, as illustrated in (Figure 2.16). Thereafter, the blocks were placed inside a plastic container containing a wet gauze, as shown in (Figure 2.17).





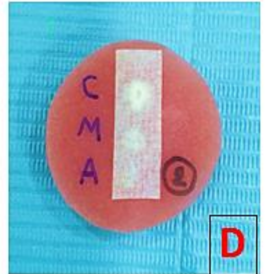


Figure 2.16 Application the medicaments for post-treatment microhardness measurement. **A**. Covering the exposed dentin by Adhesive unplasticized polyvinyl chloride leaving the canal lumen exposed.. **B**. Delivering the nitrofurantoin paste inside the root canal using an endodontic syringe **C.** Delivering the modified triple antibiotic paste inside the root canal using an endodontic syringe **D**. Covering the canal orifice by the tape.

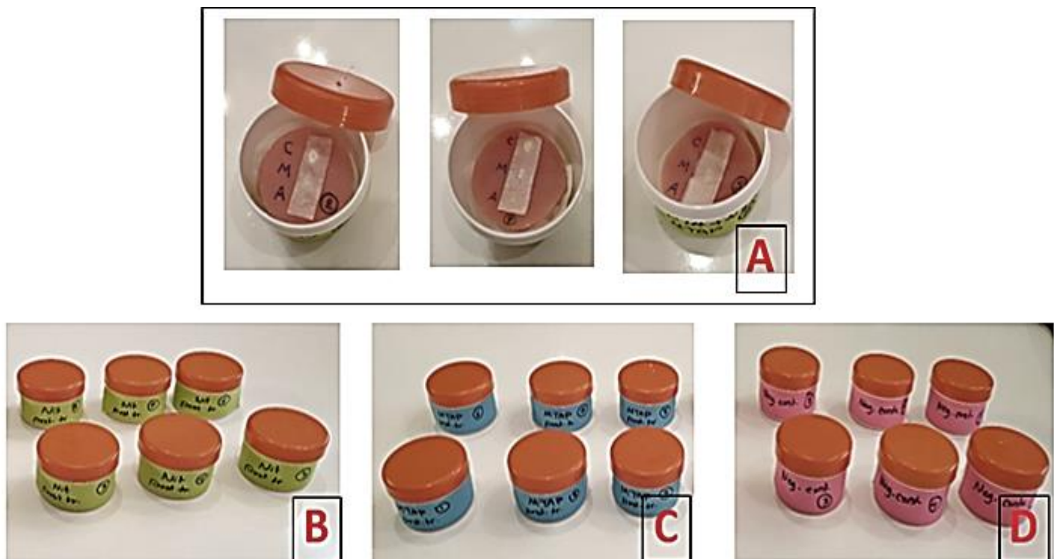


Figure 2.17 Storage of the specimens. **A**. Placing the specimen blocks inside a plastic container containing a wet gauze. **B.** Nitrofurantoin paste group. **C**. Modified triple antibiotic paste (MTAP) group. **D**. Negative control group (no medicament).

The plastic containers were stored in an incubator with approximately 100% relative humidity at 37°C for one week. After one week, all root canals were irrigated thoroughly with 5 mL of sterile distilled water for 30 seconds, using a disposable syringe with an endodontic needle, until no visible paste was observed, then the dryness of the canals was performed by using sterile paper points. After removal of the adhesive tape, post-treatment microhardness measurements were recorded on each root third adjacent to the pre-measurement (baseline indentation) to calculate the mean change in microhardness after application of the intracanal medicament.

2.2.6.2.3 Post-treatment microhardness measurement:

After application of the tested pastes, another two indentations were performed on the mesial and distal sides of each specimen third and were considered as (post-treatment) measurement. The landmark for indentation was also standardized at 500µm from the canal-dentin interface on the dentin surface and 1mm apart from the pre-treatment indentation point (Prather *et al*. 2014), as shown in (Figure 2.18).

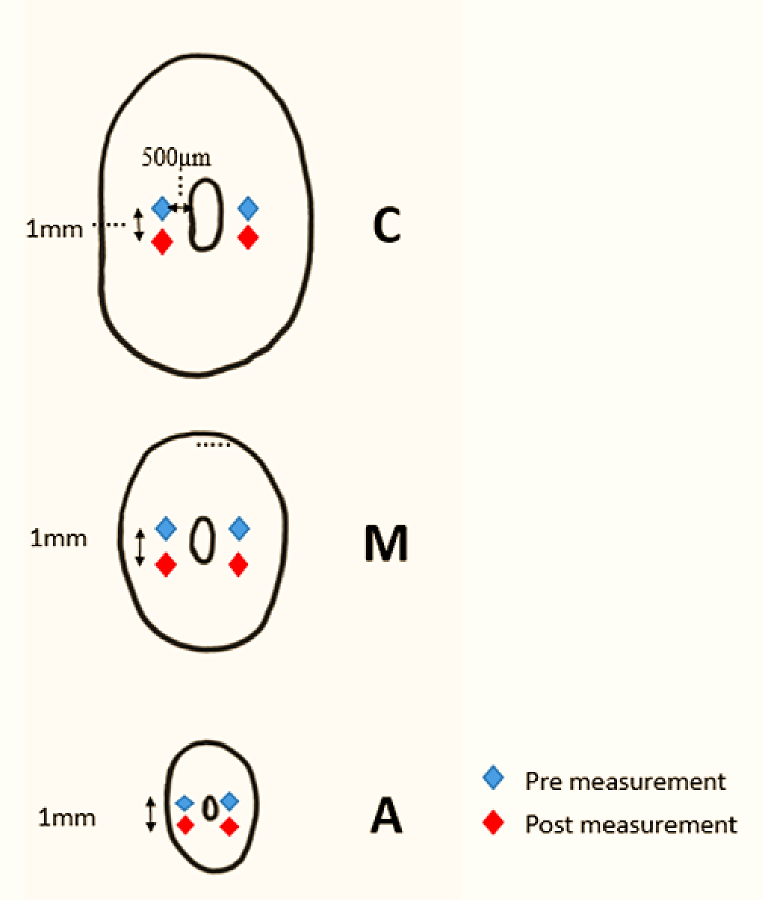


Figure 2.18 A diagram showing the three transverse sections of the three parts of the root (C= coronal third, M= middle third, A= apical third) . Blue diomand means the baseline measurement considered as pre measurement (before application of the intracanal medicament) while the red diomand indicates the post measurement (after application of the medicament), and the distance between them is 1mm.

The percentage change in microhardness of each sample was calculated as following:

$$\text{\% Change in Microhardness} = \frac{(\text{Pre-treatment microhardness} - \text{Post-treatment microhardness}) \times 100}{\text{Pretreatment microhardness.}}$$

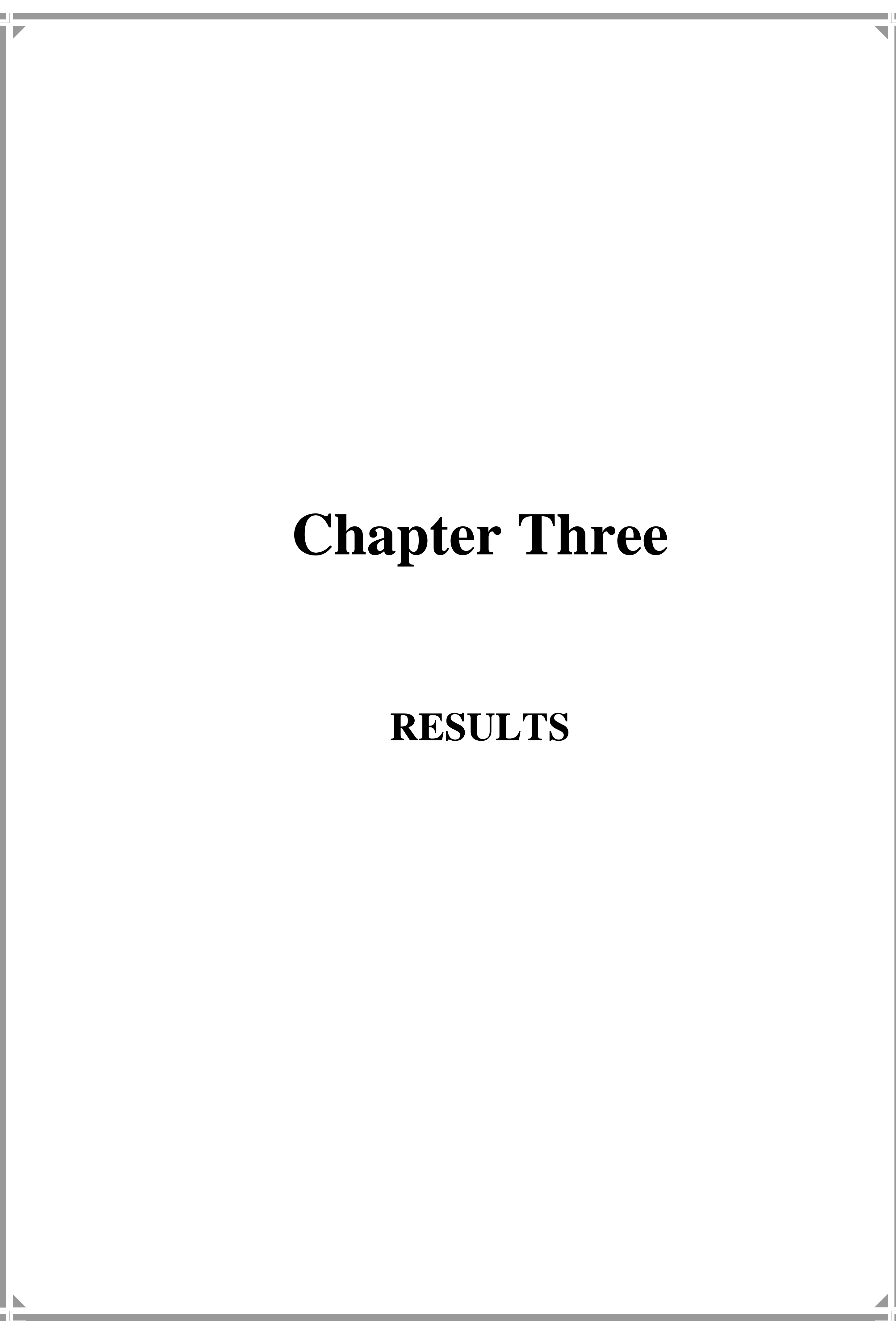
The Vickers microhardness is obtained by dividing the test force by the area of the sloping faces of the indentation. The resulting impression of the two diagonals was observed with an optical microscope and the average length of the two diagonals was measured by the built-in scaled micrometer and converted into Vickers hardness number (VHN) with the following equation:

$$HV=1854(F/D2)$$

The constant value of the equation was calculated from the specific geometry of the indenter, F= the applied load in gram force and D = the diagonals of the indentation in (µm) (Prather *et al*. 2014).

2.2.7 Statistical analyses:

The results were evaluated statistically by using the Statistical Package for the Social Sciences (SPSS) version 24.0. All the data were expressed as mean ± SD and SE. comparisons between groups were made by using the Duncan test. The Shapiro-Wilk test was used to determine normal distribution of the data. An independent t-test was used to compare the results. When the data was not normally distributed Mann-Whitney U test was used. Differences between the mean score and standard deviation of the inflammatory responses were statistically analyzed using Friedman test, while Wilcoxon test was used to compare individual pairs of groups. Changes were considered statistically significant when the P-value was 0.05 or less.



Chapter Three

RESULTS

Results

3.1 The antimicrobial investigation:

3.1.1 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of nitrofurantoin and modified triple antibiotic mixture against *Enterococcus faecalis*:

The results of MIC and MBC of nitrofurantoin and modified triple antibiotic mixture (ciprofloxacin, clindamycin, and metronidazole), in mg/mL, are presented in (Table 3.1)

Their MBC results were found to be similar to the MIC for all the three isolates of *E. faecalis*: (S1, S2, S3).

For modified triple antibiotic mixture, the MBC was (25 mg/mL) for the three isolates (S1, S2, S3), while for nitrofurantoin, the MBC was (6.25 mg/mL), (12.5 mg/mL), and (25 mg/mL) for S1, S2, and S3; respectively.

According to the CLSI, nitrofurantoin had an MIC against the three clinical isolates of *E. faecalis* (S1, S2, and S3) which demonstrate sensitive (S) result as they were ≤ 32 mg/mL. Regarding modified triple antibiotic mixture, there are no standard results to compare with according to the CLSI.

Table 3.1 MIC and MBC results (mg/mL) of nitrofurantoin and modified triple antibiotic mixture against the three isolates of *Enterococcus faecalis* (S1, S2, S3).

The Antibiotic agents	MIC- MBC		
	S1	S2	S3
Nitrofurantoin	6.25	12.5	25
Modified triple antibiotic mixture	25	25	25

3.1.2 Zone of inhibition of nitrofurantoin and modified triple antibiotic mixture against *Enterococcus faecalis*:

3.1.2.1 Group N (Nitrofurantoin):

The mean ± SD (Standard deviation) of the zone of inhibition (mm) when using different concentrations of nitrofurantoin against the three isolates of *E. faecalis* (S1, S2, and S3) is shown in (Table 3.2)

The zone of inhibition was directly proportional to the concentration of nitrofurantoin. Furthermore, it was largest in S1 and smallest in S3.

According to the CLSI, the three clinical isolates of *E. faecalis* (S1, S2, and S3) were sensitive (S) to nitrofurantoin in all concentrations (the zone of inhibition diameter is ≥ 17mm), except the concentration of (0.791 mg/mL) against (S3) which showed intermediate (I) result (the zone of inhibition diameter is 15- 16 mm).

Table 3.2 Mean ± SD of the zone of inhibition (mm) in group N, when using different concentrations of nitrofurantoin against the three isolates of *Enterococcus faecalis* (S1, S2, S3).

The Isolates	0.791 mg/mL	1.562 mg/mL	3.125 mg/mL	6.25 mg/mL	12.5 mg/mL	25 mg/mL	50 mg/mL	100 mg/mL
S1	19.16 ± 0.40 a	22.00 ± 0.57 b	24.83 ± 0.60 b	29.00 ± 0.36 c	30.00 ± 0.36 c	34.00 ± 0.44 d	38.00 ± 0.44 e	46.00 ± 0.93 f
S2	16.83 ± 0.79 a	19.83 ± 0.87 a	23.16 ± 0.70 b c	26.16 ± 0.65 b	27.83 ± 0.60 c d	30.16 ± 0.40 d	33.83 ± 0.83 e	37.83 ± 0.60 f
S3	14.83 ± 0.70 a	17.83 ± 0.70 b	21.16 ± 0.47 c	24.00 ± 0.81 d	26.16 ± 0.47 d e	28.00 ± 0.44 e f	29.83 ± 0.30 f	34.00 ± 0.63 g

Different letters indicate significant differences at $p < 0.05$.

3.1.2.2 Group M (MTAP):

The mean ± SD of the zone of inhibition (mm), when using different concentrations of modified triple antibiotic mixture against the three isolates of *E. faecalis* (S1, S2, and S3) is presented in (Table 3.3)

The zone of inhibition was directly proportional to the concentration of triple antibiotic mixture. Additionally, it was largest in S1 and smallest in S3. There are no standard results for modified triple antibiotic mixture to compare with according to the CLSI.

Table 3.3 Mean ± SD of the zone of inhibition (mm) in (group M) when using different concentrations of modified triple antibiotic mixture against three isolates ins of *Enterococcus faecalis* (S1, S2, S3).

The Isolates	0.791 mg/mL	1.562 mg/mL	3.125 mg/mL	6.25 mg/mL	12.5 mg/mL	25 mg/mL	50 mg/mL	100 mg/mL
S1	14.00 ± 0.57 a	16.16 ± 0.30 b	20.00 ± 0.57 c	22.00 ± 0.51 c	26.00 ± 0.57 d	29.16 ± 0.47 e	30.83 ± 0.30 e	33.83 ± 0.30 f
S2	13.00 ± 0.36 a	16.00 ± 0.36 b	18.00 ± 0.73 c d	19.83 ± 0.47 c d	20.00 ± 0.44 c d	22.00 ± 0.57 d	25.00 ± 0.57 e	28.00 ± 0.51 f
S3	11.00 ± 0.51 a	13.00 ± 0.36 b	15.16 ± 0.30 c	18.00 ± 0.57 d	20.00 ± 0.25 e	22.16 ± 0.30 f	24.00 ± 0.36 g	26.00 ± 0.36 h

Different letters indicate significant differences at $p < 0.05$.

The comparison between the mean of the zone of inhibition in mm according to the MBC of nitrofurantoin and modified triple antibiotic mixture against the three isolates of *E. faecalis*; (S1, S2, S3) is shown in (Table 3.4).

The diameter of zone of inhibition of nitrofurantoin was smaller than that of triple antibiotic mixture against (S1) and statistically non-significant difference was found between them ($p>0.05$).

While, the diameter of the zone of inhibition of nitrofurantoin was more than that of triple antibiotic mixture against (S2) and (S3), and the difference between them was statistically highly significant ($p<0.001$).

Table 3.4 Comparison between the mean of the zone of inhibition (mm) according to the MBC of modified triple antibiotic mixture and nitrofurantoin, against the three isolates of *Enterococcus faecalis*; (S1, S2, S3).

The Isolates	MBC Modified triple antibiotic mixture	Zone of inhibition Modified triple antibiotic mixture	MBC Nitrofurantoin	Zone of inhibition Nitrofurantoin	p-value
S1	25 mg/mL	30.16 ± 0.47	6.25 mg/mL	29.00 + 0.36	0.06
S2	25 mg/mL	22.00 ± 0.57	12.5 mg/mL	27.83 + 0.60	0.001
S3	25 mg/mL	22.16 ± 0.30	25 mg/mL	28.00 + 0.44	0.001

An independent T-test of two means was used.

3.2 Viable bacterial count (CFU) in ex-*vivo* assay:

3.2.1 Group N (Nitrofurantoin paste):

The mean ± SD results of viable bacterial count in (CFU) of the three isolates of *E. faecalis* of this group with different concentrations of nitrofurantoin, and the p-value between CFU of canal lumen and dentinal chips is shown in (Table 3.5).

In this group, nitrofurantoin was used at different concentrations (6.25, 12.5, 25, 50, and 100 mg/mL) against the three isolates of *E. faecalis*; (S1, S2, and S3) and the CFU was counted.

Isolate S1: There was no viable bacteria seen when using nitrofurantoin at concentrations: (6.25, 12.5, 25, 50, and 100 mg/mL).

Isolate S2: There was no viable bacteria found when using nitrofurantoin at (12.5, 25, 50, 100 mg/mL). While when using nitrofurantoin at a concentration of (6.25mg/mL), CFU was found, and the CFU of *E. faecalis* from the canal lumen was less than the CFU of *E. faecalis* in the dentinal chips, and the difference was statistically significant ($p<0.05$).

Isolate S3: There was no viable bacteria found when using nitrofurantoin at (25, 50, 100 mg/mL). While when using nitrofurantoin at a concentration of (6.25mg/mL), CFU was noted, and the CFU of *E. faecalis* from the canal lumen was less than that of dentinal chips, and the difference was statistically significant ($p=0.05$).

On the other hand, using nitrofurantoin at a concentration of 12.5 mg/mL, viable bacterial count was seen, and the CFU of *E. faecalis* from the canal lumen was less than that of dentinal chips, and the difference was statistically significant ($p<0.05$).

Table 3.5 The mean ± SD results of viable bacterial count (CFU) of the three isolates of *Enterococcus faecalis* when using nitrofurantoin paste in different concentrations. P-value to compare between the viable bacterial count of canal lumen and dentinal chips.

The Isolates	Site of the sample of bacteria	Concentration of nitrofurantoin (mg/mL)						
		6.25	p-value	12.5	p-value	25	50	100
S1	Canal lumen	0	-	0	-	0	0	0
	Dentinal chips	0		0		0	0	0
S2	Canal lumen	283.33 ± 47.72	0.028	0	-	0	0	0
	Dentinal chips	433.33± 33.33		0		0	0	0
S3	Canal lumen	366.66 ± 49.44	0.05	266.66 ± 42.16	0.11	0	0	0
	Dentinal chips	516.66 ± 47.72		400.00 ± 63.24		0	0	0

3.2.2 Group M (MTAP):

The mean ± SD results of viable bacterial count of the three isolates of *E. faecalis* of this group with different concentrations of MTAP, and the p-value between viable bacterial count of canal lumen and dentinal chips is shown in (Table 3.6).

In this group, MTAP was used at different concentrations (6.25, 12.5, 25, 50, and 100 mg/mL) against the three isolates of *E. faecalis*; (S1, S2, and S3) and the CFU was counted.

Isolate S1: There was no viable bacterial count seen when using MTAP at concentrations: (25, 50, and 100 mg/mL). While when using MTAP at a lower concentration of (6.25 mg/mL), the CFU was recorded and the result of CFU of *E. faecalis* in canal lumen was less than CFU of *E. faecalis* in dentinal chips, and the difference was statistically significant ($p<0.05$). Also when using MTAP at a concentration of (12.5, mg/mL), the CFU was recorded, and the result of CFU of *E. faecalis* in canal lumen was less than viable bacterial count in dentinal chips and the difference was statistically non-significant ($p>0.05$).

Isolate S2: There was no viable bacterial count found when using MTAP at concentrations: (25, 50, and 100 mg/mL). While when using MTAP at a concentration of (6.25 mg/mL), the CFU was recorded and the result of CFU of *E. faecalis* in canal lumen was less than CFU in dentinal chips, and the difference was statistically non-significant ($p>0.05$). Also using MTAP at a concentration of (12.5, mg/mL), the CFU was recorded, and the result of viable bacterial count in canal lumen was less than viable bacterial count in dentinal chips and the difference was statistically non-significant ($p>0.05$).

Isolate S3: There was no CFU seen when using MTAP at concentrations: (25, 50, and 100 mg/mL). While when using MTAP at a concentration of (6.25 mg/mL), the CFU was recorded and the result of CFU of *E. faecalis* in canal lumen was less than CFU in dentinal chips and the difference was statistically non-significant ($p>0.05$). Also using MTAP at a concentration of (12.5, mg/mL), the CFU was recorded, and the result of CFU of *E. faecalis* in canal lumen was less than viable bacterial count in dentinal chips and the difference was statistically non-significant ($p>0.05$).

Table 3.6 The mean ± SD results of viable bacterial count (CFU) of the three isolates of *Enterococcus faecalis* when using MTAP in different concentrations. P-value to compare between the viable bacterial count of canal lumen and dentinal chips.

The Isolates	Site of sample of bacteria	Concentration of MTAP (mg/Ml)						
		6.25	p-value	12.5	p-value	25	50	100
S1	Canal lumen	183.33± 30.73	0.016	66.66 ± 33.33	0.120	0	0	0
	Dentinal chips	300.0± 25.81		191.33± 60.09		0	0	0
S2	Canal lumen	271.47 ± 54.26	0.095	200.00 ± 51.63	0.52	0	0	0
	Dentinal chips	416.66 ± 47.72		250.00 ± 56.27		0	0	0
S3	Canal lumen	533.33± 42.16	0.068	333.33± 175.11	0.1	0	0	0
	Dentinal chips	683.33± 60.09		500.00± 57.73		0	0	0

3.2.3 Group W (Distilled water):

The viable bacteria were counted when using distilled water as a negative control. viable bacterial count was seen in all the samples of the three isolates of *E. faecalis* (S1, S2, and S3). Likewise, the resulting viable bacterial count in canal lumen was less than the viable bacterial count in dentinal chips, and the difference was statistically significant ($p<0.05$) between them for the three isolates, as shown in (Table 3.7).

Table 3.7 The mean ± SD results of CFU of the three isolates of *Enterococcus faecalis* when using distilled water. P-value to compare between the viable bacterial count of canal lumen and dentinal chips.

The isolate	Site of sample of bacteria	Mean ± SD	P-value
S1	Canal lumen	27000.0000 ± 3510.74381	0.01
	Dentinal chips	39500.0000 ± 4847.3785)	
S2	Canal lumen	28283.3333 ± 8584.80893	0.013
	Dentinal chips	46500.0000 ± 5875.08865	
S3	Canal lumen	57741.9165 ± 4885.35226	0.02
	Dentinal chips	64333.3333 ± 6468.72819	

3.3 Biocompatibility of nitrofurantoin paste and MTAP in rats' subcutaneous connective tissue:

3.3.1 Statistical analysis of the inflammatory scores:

The mean values of histological scores in different groups is shown in (Table 3.8). Statistically significant differences were found between sites on 3rd ,7th, 14th, and 30th days following implantation of TG1 ($p<0.05$), highly significant in group TG2 and PC ($p<0.001$), while the difference was statistically non-significant in NC ($p>0.05$).

The p-values for comparisons between the groups in all of the four-period intervals is presented in (Table 3.9). Statistically significant difference in inflammatory response was found between PC and TG1 in 3,7, and 14 day intervals ($p<0.05$) but non-significant at day 30 ($p>0.05$). While the difference in inflammatory response between PC and TG2 was statistically significant at day 3 and 7 ($p<0.05$), then the difference became non-significant in day 14 and 30 ($p>0.05$). The intensity of inflammatory response was more in TG2 than TG1 in all period intervals but the difference

was statistically non-significant ($p>0.05$). The intensity of inflammatory response in TG1 and TG2 were more than NC in all time intervals and the difference was non-significant ($p>0.05$) except at day 3 and 7 in TG2, the difference was statistically significant ($p<0.05$) Meanwhile, PC demonstrated significant differences with NC in all four-period intervals ($p<0.05$). The means plot for the groups with time periods (day3, day7, day14 and day30) is shown in (Figures 3.1).

Table 3.8 Mean ± SD of the histological scores of the inflammatory response after 3, 7, 14, and 30 days following implantation for all four groups (TG1, TG2, PC, and NC).

Group	Mean ± SD					p-value
	Day 3	Day 7	Day 14	Day 30	Total	
TG1	1.33 ± 0.51	1.0 ± 0.63	0.5 ± 0.54	0.33 ± 0.51	0.79 ± 0.65	0.02
TG2	1.67 ± 0.51	1.17 ± 0.75	0.67 ± 0.51	0.33 ± 0.51	0.96 ± 0.65	0.004
PC	2.33 ± 0.51	1.83 ± 0.75	1.17 ± 0.40	0.83 ± 0.75	1.54 ± 0.83	0.002
NC	0.83 ± 0.40	0.5 ± 0.54	0.33 ± 0.51	0.17 ± 0.40	0.46 ± 0.5	0.12

Table 3.9 P-value to compare between the four groups (TG1, TG2, PC, and NC), in all experimental period of time (3, 7, 14, and 30 days).

Groups	p- values			
	Day 3	Day 7	Day 14	Day 30
PC- TG1	0.014 c	0.025 c	0.046 c	0.083 a
PC- TG2	0.046 c	0.046 c	0.083 a	0.083 a
TG1- TG2	0.151 b	0.317 b	0.317 b	1 b
TG1- NC	0.083 a	0.083 a	0.317 b	0.317 b
TG2 - NC	0.025 c	0.046 c	0.157 b	0.317 b
PC - NC	0.024 c	0.023 c	0.025 c	0.046 c

*Different letter shows different statistical associations.

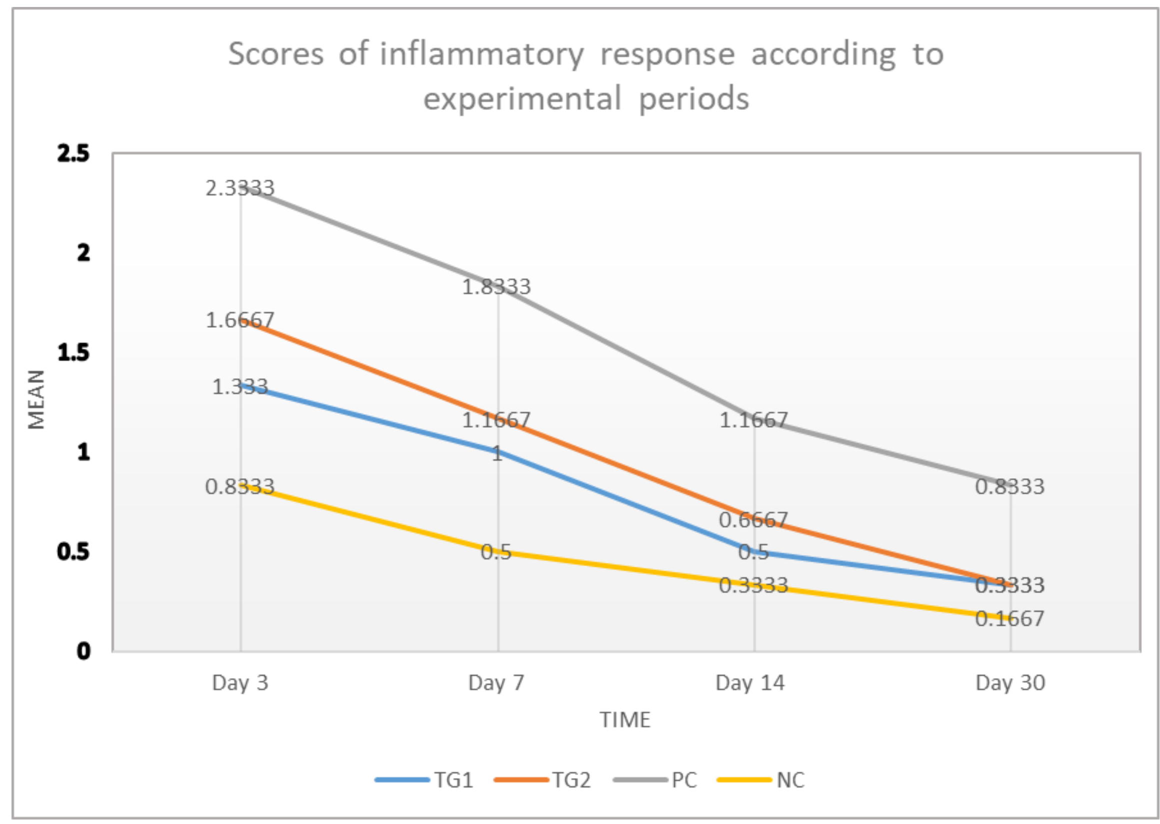


Figure 3.1 Means plot for the groups (TG1, TG2, PC, and NG) with time periods (Day3, Day7, day14 & Day30).

3.3.2 Histopathological evaluations and inflammatory scoring of cutaneous wound healing for the groups:

3.3.2.1. Group TG1 (Testing group1): 12.5 mg/mL nitrofurantoin paste.

- On day three: There was a clear bridge between scab and dermis. Also, the formation of the demarcation line was observed under the tissue necrosis. If it was compared to the control group, the wound partially closed and mild-moderate degree of inflammatory cell infiltration in epidermis migrates to dermis with the presence of newly formed blood vessels and fibroblast proliferation. (Figure 3.2-A)

- On day seven: Histologically, healing became more pronounced and showed an early phase of the re-epithelization process. The thickness of the epidermis was slightly similar to that of the intact epidermis and showed immature-hyperplastic and disorganized epidermis overlying the area of the wound, with an increase in the thickness of the dermal layer by the presence of well-arranged mature collagen fibers as a bundle with fibroblast proliferation. (Figure 3.2-B)

- On day fourteen: The wound showed remodeling phase, and re-epithelialization was markedly complete. Tissue regeneration had better quality, with a normal epidermis covering the wound area; collagen fibers were thicker and denser. Some cutaneous annexes, such as sebaceous glands and hair follicles in the center of the scar tissue, were formed but at a different level in each group. At this stage, the wounded skin regained its strength and elasticity and advances to the final reconstruction of the dermis by reorganization of collagen and elastic fibers. (Figure 3.2-C)

- On day thirty: Histologically, in the TG1 group, the wound showed a late stage of regenerating phase with complete maturation of the wound, and the final steps of dermal reorganization are underway but with variable levels. Also, in the TG1 group, the mature wound showed well-organized epidermis with dermal papillae development, while the scar intensity ranged from a mild-moderate and new blood vessel formations, and few numbers of cutaneous annexes were observed, with well-organized, thick bundles of collagen fibers. (Figure 3.2-D)

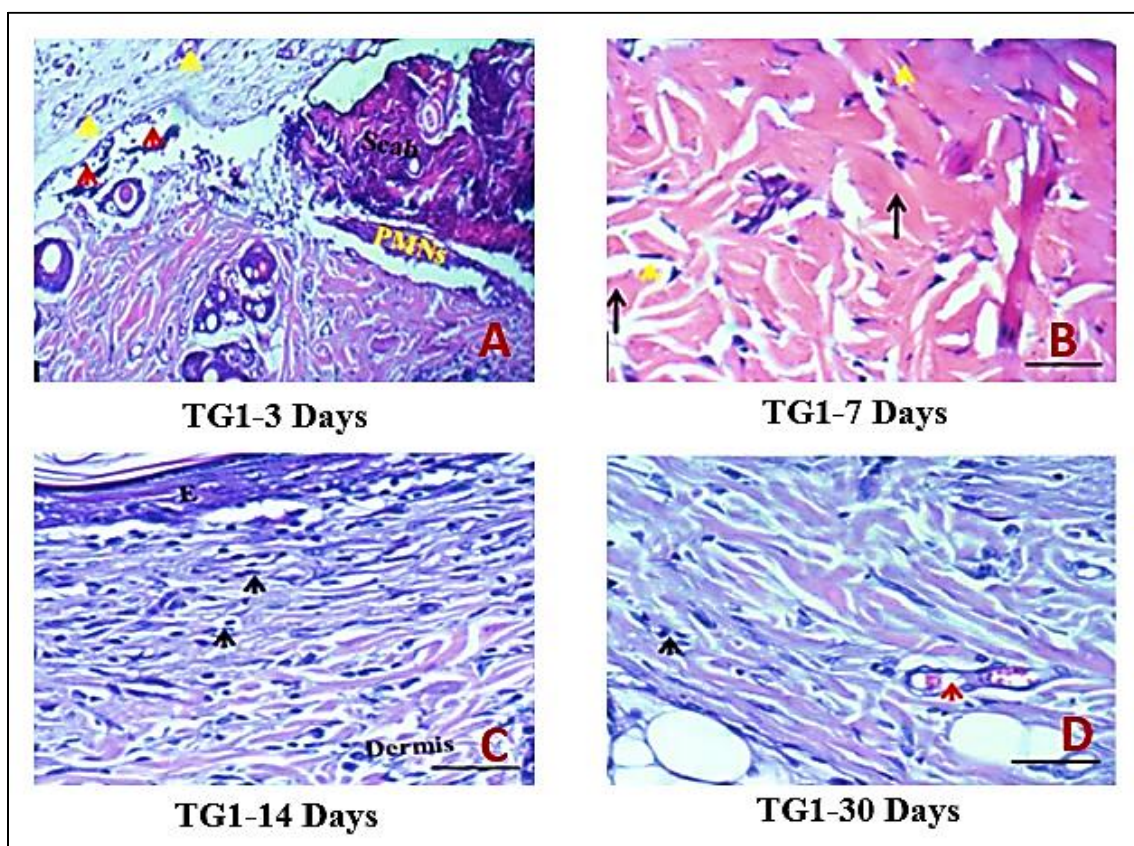


Figure 3.2 Slides showing the inflammatory response of the connective tissue to the 12.5 mg/mL Nit (TG1) at days 3, 7, 14, and 30 respectively. (**A**) shows mild-moderate degree inflammatory cells as line demarcation between dermis and the wound, (**B**) shows increased in thickness of dermal layer by mature bundle of collagen fibers (black arrows) with proliferative fibroblasts as indicated by yellow arrows, (**C**) shows infiltration of inflammatory cells (black arrows), well-organized thick bundles of collagen fibers observed in dermis, and (**D**) shows mild inflammatory cells infiltration (black arrows) and blood vessels (red arrows) formation, and few numbers of cutaneous annexes, well-organized thick bundles of collagen fibers.

3.3.2.2 Group TG2 (Testing group 2): 25 mg/mL Nitrofurantoin paste.

- On day three: The wound was partially filled by the fibrin network which was in the incisional space. This network contained blood cells and created a scaffold for migrating fibroblasts. Also, clear epidermis formation has been observed. The wound incision was rich in inflammatory cells (PMNs);

thus, the demarcation line was formed and separated the necrosis from vital tissue with a moderate degree of inflammatory cell infiltration in epidermis and dermis. Vessel congestion beneath the dermis was possible, to observe the beginning of neo-angiogenesis with slightly fibroblast proliferation, and the epidermis started to regenerate. (Figure 3.3-A)

- <u>On day seven</u>: Histologically, there were features of a healed wound in the TG2 group, as demonstrated by the cessation of the inflammatory process. The inflammatory phase was almost complete, and the thickness of the wound was similar to the intact wound. It appeared in the proliferative phase, including immature-hyperplastic and disorganized epidermis overlying the area of the wound with fewer inflammatory cells in dermis and hypodermis. (Figure 3.3-B)

- <u>On day fourteen</u>: A new epidermis with an uneven organization of layers was formed with a mild thickness of keratinization but marked density of scar tissue (granulation tissues beneath all wounds had matured to form scar tissues), and inflammatory cells in scar tissue were observed in the center of the wound. In the dermal layer, well-organized thick bundles of collagen fibers were observed. The wounded skin was completely enclosed with the well-uniform developed epidermis and organized tissue layers, with the moderate thickness of keratinization. Additionally, moderate density of scar tissue was loaded with large numbers of blood vessels and inflammatory cell (neutrophils, macrophages, lymphocytes, and fibroblasts), which were seen in the center of the wound, the dermis having various sizes of thick, uniform, compact, and regularly arranged collagen fibers. (Figure 3.3-C)

- <u>On day thirty</u>: The mature wound showed a well-organized epidermis with mild scar intensity and the presence of few inflammatory cells and blood vessel formations; also, the numbers of cutaneous annexes, such as sebaceous glands and hair follicles, were increased, with well-organized thick bundles of collagen fibers. (Figure 3.3-D)

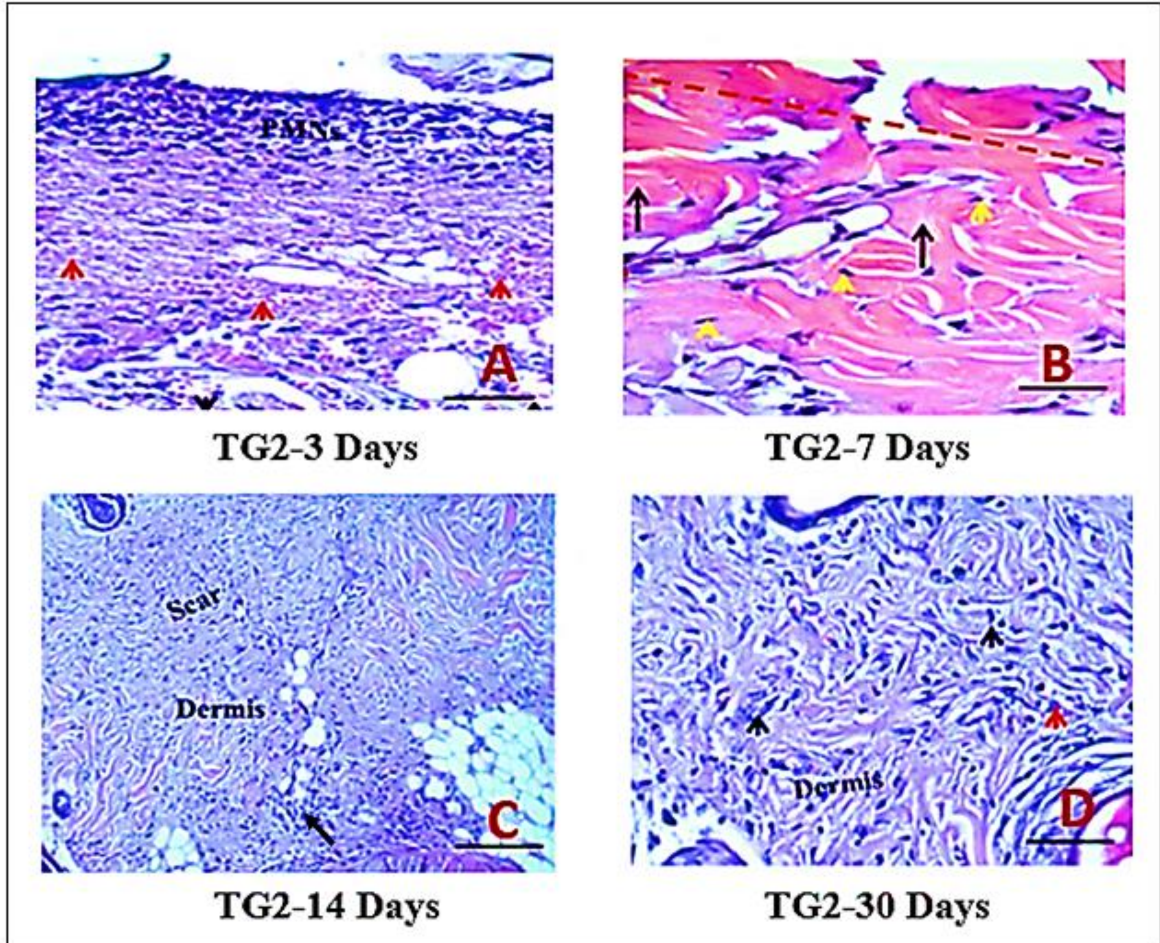


Figure 3.3 Slides showing the inflammatory response of the connective tissue to the 25 mg/mL Nit (TG2) at days 3, 7, 14, and 30 respectively. (**A**) shows polymorphonuclear cell infiltration, congested blood vessels (red arrows) with angiogenesis as indicated by black arrows; (**B**) shows early re-epithelization by forming immature disorganized epidermis (red dash line), increased in thickness of dermal layer by extreme, mature bundle of collagen fibers (black arrows) with proliferative fibroblasts as indicated by yellow arrows; (**C**) shows inflammatory cells (black arrows), and large numbers of blood vessels (red arrows); and (**D**) shows minimum inflammatory cells infiltration (black arrows) and blood vessels (red arrows) and increased the numbers of cutaneous annexes, well-organized thick bundles of collagen fibers.

3.3.2.3 Group PC (Positive Control): 25mg/ml MTAP

- On day three: The formation of the demarcation line was evident. The regeneration of the epidermis was apparent; furthermore, fibrin and PMN cells bridged the whole incision, with a huge infiltration of inflammatory cells in the dermis and hypodermis with proliferation and migration of fibroblasts. Angiogenesis was observed, but the number of fibroblasts increased in the dermis near the wounded area with new collagen formation. (Figure 3.4 -A)

- On day seven: Histologically, cutaneous incisions in the PC group showed a typical histological picture of the proliferative phase with complete regression of the inflammatory phase. The inflammatory cells were distributed and showed an expressive representation of fibroblasts and new vessels. Furthermore, the re-epithelization occurred from hair follicles and immature-disorganized hyperplastic epidermis overlying the area of the wound was observed. At the layer of the dermis, fibroblasts were predominantly vertically oriented. (Figure 3.4 -B)

- On day fourteen: The remodeling phase showed keratinized substances which had accumulated and the thickness of the stratified squamous epithelium was even and well developed, while the density of scar tissue was mild, with large numbers of blood vessels and inflammatory cells. (Figure 3.4 -C)

- On day thirty: Histologically, the wound revealed minimum scar tissue intensity with few numbers of inflammatory cells and blood vessels, while highly-increased numbers of cutaneous annexes were seen with well-organized thick bundles of collagen fibers in the dermal layer. (Figure 3.4 -D)

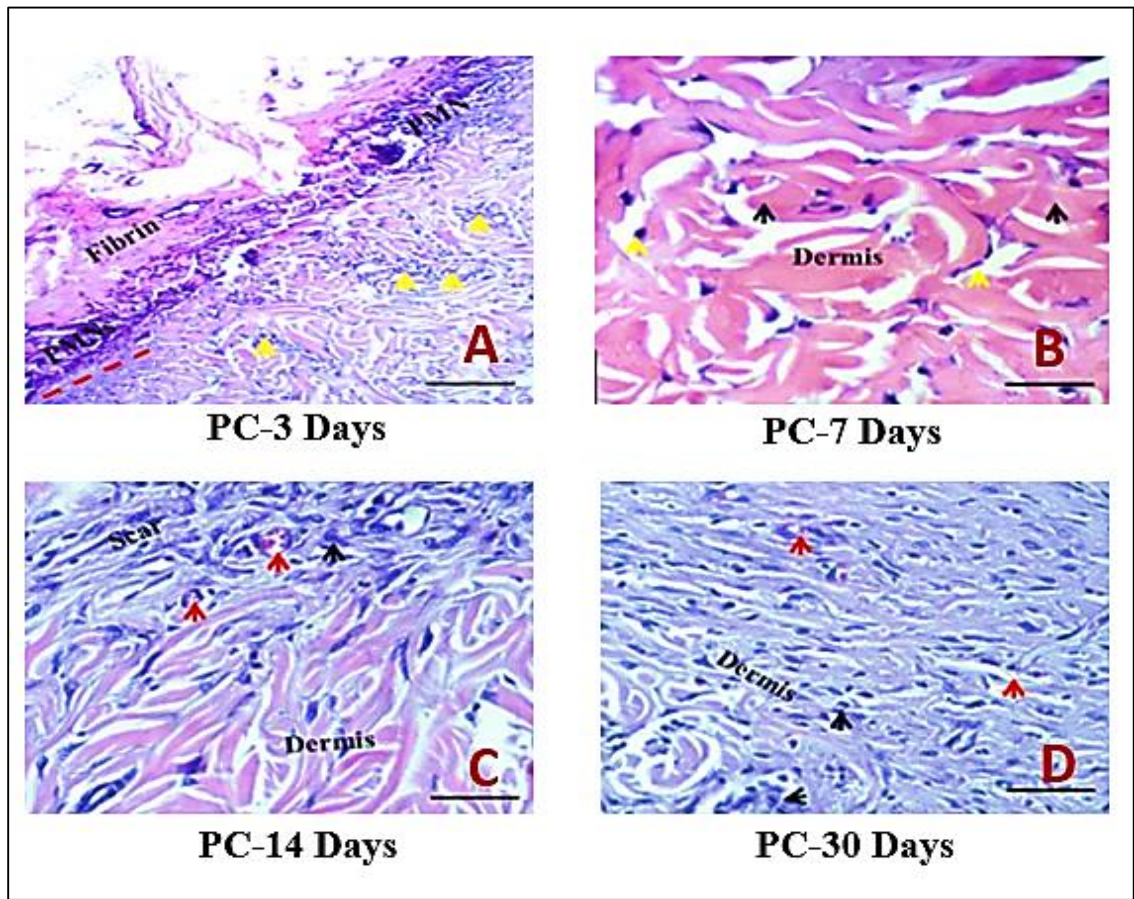


Figure 3.4 Slides showing the inflammatory response of the connective tissue to the 25 mg/ml MTAP Positive Control (PC) on days 3, 7, 14, and 30 respectively. (**A**) shows the fibrin and PMN cells bridged the whole incision, the epidermis formed (red dash line), with a huge infiltration of inflammatory cells in the dermis and hypodermis (yellow arrows); (**B**) shows increased thickness of the dermal layer by intense, well arranged mature bundles of collagen fibers (black arrows) with proliferative fibroblasts as indicated by yellow arrows; (**C**) shows mild inflammatory cells (black arrows), and large numbers of blood vessels (red arrows) having thick, compact and regularly arranged collagen fibers observed in the dermis; while (**D**) is showing little inflammatory cell infiltration (black arrows) and blood vessel (red arrows) formation, highly increased numbers of cutaneous annexes, and well organized thick bundles of collagen fibers.

3.3.2.4 Group NC (Negative Control): Empty tube

- On day three: Histologically the cutaneous wound did not close completely, with a scab formed by necrotic tissue remnants, fibrin, polymorphonuclear cell infiltration, and inflammatory cell infiltration without an epithelial layer under the scab. Vessels were congested in the dermis, and there were no hair follicles, sebaceous or sweat glands, no obvious signs of new capillary formation and fibroblast proliferation. (Figure 3.5-A)

- On day seven: There was a granulation phase or inflammatory phase on the surface wound area. Necrosis of skin tissue was noted as a consequence of mechanical damage. A continuous layer of marked granulation (inflammatory cell, collagen deposition, and angiogenesis) across the entire wound gap and depth, in which the thickness of the granulation tissues was more in the surface layer than in the dermal layer.

Additionally, collagen fibers were seen in a disorganized arrangement, placed as small, randomly-distributed fibrils with extremely thin-walled blood vessels (angiogenesis), and were distributed throughout the granulation tissue, while the intense inflammatory reaction (inflammatory cells including neutrophils, macrophages, lymphocytes, plasma cells, and fibroblasts) was dominant. (Figure 3.5-B).

- On day fourteen: Wound healing was more progressive, on-going re-epithelialization (proliferative phase) and granulation were observed in the NC group. The PMNs decreased while the wound thickness increased, and they were covered by a very small scab. The larger size and dark nuclear-stained fibroblasts appeared with a large number of thick bundles of disorderly collagen fibers filled the inter-cellular matrix. (Figure 3.5-C)

- <u>On day thirty:</u> The wound healing showed an early regeneration phase; wounds are fully re-epithelialized with keratinization, scar-tissue formed with marked intensity and loaded with the new blood vessel formation, with infiltration of inflammatory cells (neutrophils, macrophages, lymphocytes, and fibroblasts). In the dermis, few cutaneous annexes such as sebaceous glands and hair follicles were formed, and well organized, thick bundles of collagen fibers were also observed. (Figure 3.5-D)

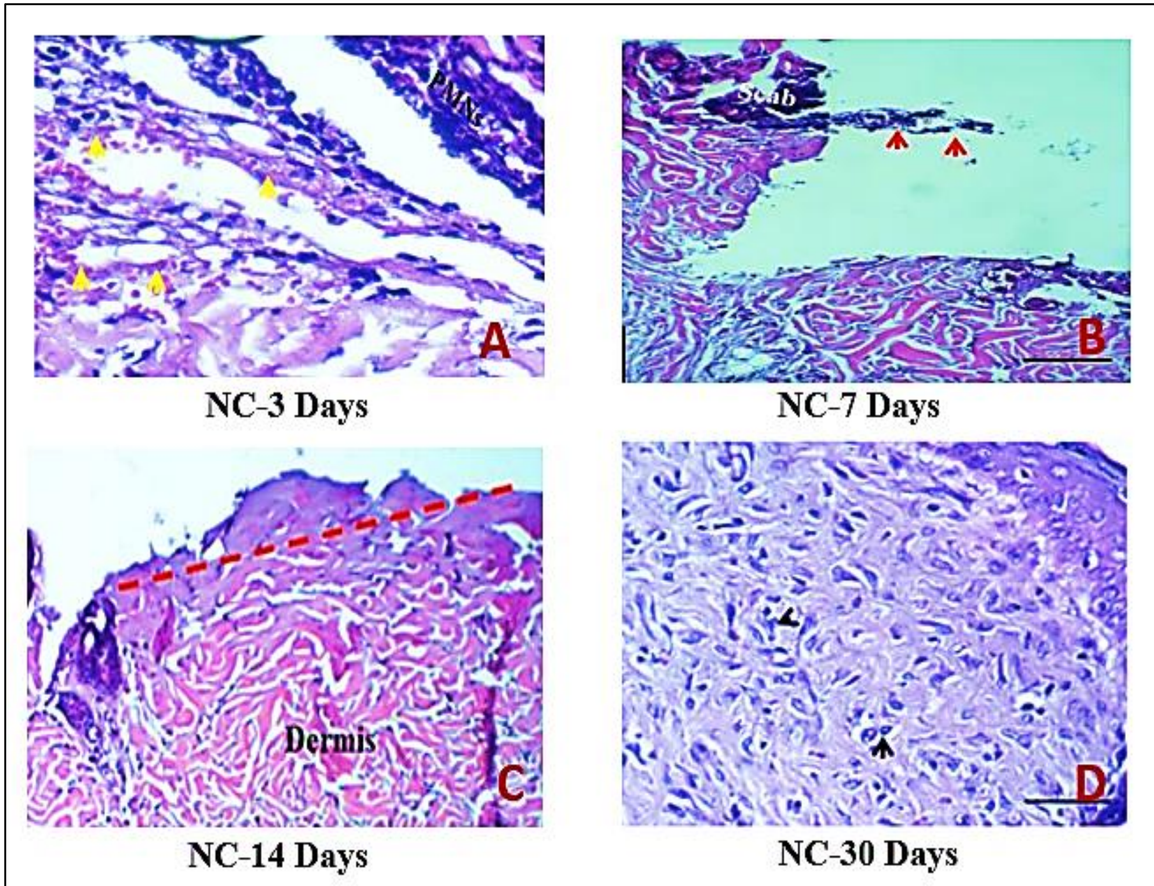


Figure 3.5 Slides showing the inflammatory response of the connective tissue to the empty tube, (NC) at days 3, 7, 14, and 30 respectively. (**A**) shows infiltration of neutrophils in the dermis with congested blood vessels and free RBC (yellow arrows), and (**B**) shows the wound surface covered with scab and necrotic debris (red arrows). Slide (**C**) shows a typical hyperplastic-immature epidermis as indicated by red dash lines with well-organized collagen fibers in the dermal layer, and few neovascularizations, while (**D**) shows the completely mature wound and the late stage of the remodeling phase, a well organized epidermis, and minimum inflammatory cell infiltration (black arrows).

3.3.3 pH measurement:

The average pH result of nitrofurantoin solution with a concentration of (12.5mg/mL) and (25 mg/mL) were (7.0) and (7.1) respectively, which are neutral. While, the average pH of modified triple antibiotic solution with a concentration of (25 mg/mL) was (4.9), which is acidic, as shown in (Figure 3.6).

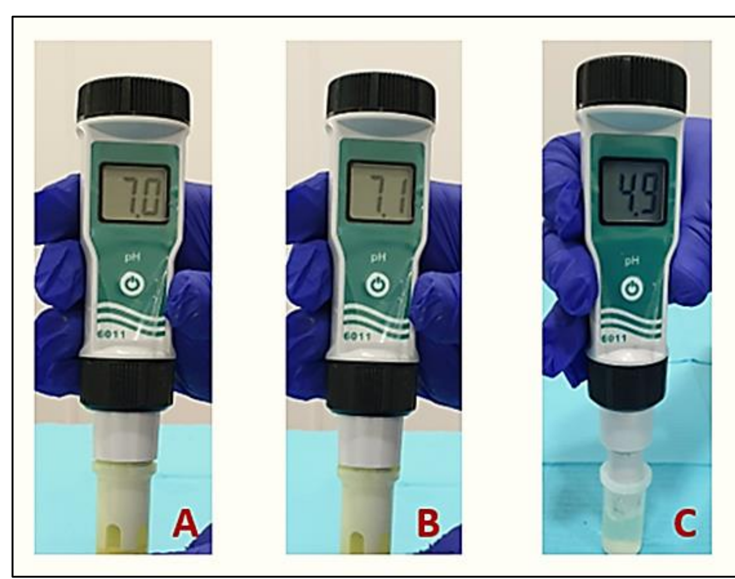


Figure 3.6 The pH result of the medicaments. **A.** pH=7.0 for 12.5 mg/mL nitrofurantoin. **B.** pH=7.1 for 25 mg/mL nitrofurantoin. **C.** pH=4.9 for 25 mg/mL MTAP.

3.4 Microhardness evaluation of the radicular dentin after using nitrofurantoin paste and MTAP.

The mean $\pm$ standard deviation (SD) of the microhardness values (HV) of each root third in the three groups as measured in pre-treatment samples is illustrated in (Table 3.10). The microhardness values (HV) being highest in the apical third and decrease to be the lowest values in the coronal third and the differences among all groups in all three thirds were significant ($p < 0.05$), and non-significant different between the groups in the same third of the root ($p > 0.05$).

In post-treatment, also the (HV) being highest in the apical third and decrease to be the lowest values in the coronal third and the differences were significant ($p < 0.05$) for each group. Moreover, regarding regional variation according to the root thirds after application of the medicament, at the coronal third, although MTAP showed more reduction in (HV) than nitrofurantoin, both of them showed a significant difference in the reduction compared with control group ($p < 0.05$), Whereas, concerning the other two thirds of the root (middle and apical), the reduction in (HV) was statistically non-significant between nitrofurantoin and control group ($p > 0.05$), in contrast, MTAP showed a statistically significant difference ($p < 0.05$). When comparing between nitrofurantoin and MTAP, at all the root third, the difference was statistically significant between them ($p < 0.05$), as shown in (Table 3.11).

The mean $\pm$ SD of the microhardness value pre-treatment and post-treatment is shown in (Table 3.12). In post-treatment, nitrofurantoin and MTAP groups showed a significant reduction in the (HV) when compared with pre-treatment microhardness values ($p < 0.001$), but non-significant for control group ($p>0.05$).

The percent reduction in microhardness values (HV), along with their standard deviations ($\pm$ SD), for the three groups is presented in (Table 3.13). At all the root third, MTAP showed more percentage reduction in (HV) (9.18 %) than nitrofurantoin (1.88 %), and the different was statistically significant when compared with control groups ($p<0.0001$). Concerning the root third, at the coronal third, MTAP reduced (HV) about (13.73%) more than the middle (8.72%) and apical third (5.07 %). While, nitrofurantoin reduced the (HV) about (4.10%) at the coronal third then the percentage of reduction being less at the middle and apical third, which were (1.16% and 0.36 %) subsequently.

Microhardness value (HV) of the three root thirds (coronal, middle and apical) for the three groups in post-treatment (control. nitrofurantoin and MTAP) is illustrated in (Figure 3.7).

Reduction in (HV) in post-treatment of the three different groups compared to the pre-treatment microhardness, in all of the three thirds of the root is shown in (Figure 3.8).

While, (Figure 3.9) shows mean percentage (%) of microhardness reduction of radicular dentin after application of nitrofurantoin and MTAP.

Table 3.10 Mean ± SD of the microhardness values (HV) with comparison between the groups and the root thirds in pre-treatment.

Groups	Apical	Middle	Coronal
Nit	52. 04 ± 0.23 a A	48. 19 ± 0.23 a B	46.07 ± 0.19 a C
MTAP	52.02 ± 0.03 a A	48. 16 ± 0.20 a B	46. 09 ± 0.16 a C
DW	52.05 ± 0.08 a A	48. 18 ± 0.16 a B	46. 08 ± 0.13 a C

*Similar lower-case letters indicate non-significant difference between the groups in the same third of the root.

*Different upper-case letters indicate significant difference between the three thirds of the roots in the same group.

Table 3.11 Mean ± SD of the microhardness values (HV) with comparison between the groups and the root thirds in post-treatment.

Groups	Apical	Middle	Coronal
Nit	51. 85 ± 0.25 a A	47. 633 ± 0.26 a B	44.18 ± 0.25 a C
MTAP	49.38 ± 0.14 b A	43. 96 ± 0.20 b B	39. 75 ± 0.24 b C
DW	52.05 ± 0.08 a A	48. 18 ± 0.16 a B	46. 08 ± 0.13 c C

*Different lower-case letters indicate significant difference between the groups in the same third of the root.

*Different upper-case letters indicate significant difference between the three thirds of the roots in the same group.

Table 3.12 Mean ± SD of the microhardness values (HV) of the radicular dentin pre/ post-treatment for each root third of the three groups.

Groups	Apical		Middle		Coronal	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Nit	52. 04 ±0.23	51. 85 ± 0.25*	48. 19 ± 0.23	47. 63 ± 0.26*	46.07 ± 0.19	44.18 ± 0.25*
MTAP	52.02 ± 0.03	49.38 ± 0.14*	48. 16 ± 0.20	43. 96 ± 0.20*	46. 09 ± 0.16	39. 75 ± 0.24*
Control	52.05 ± 0.08	52.05 ± 0.08	48. 18 ± 0.16	48. 18 ± 0.16	46. 08 ± 0.13	46. 08 ± 0.13

* $p<0.05$ compared to pretreatment.

Table 3.13 Mean + SD and percentage reduction in microhardness values (HV) of the radicular dentin of the groups.

Root thirds	Apical	Middle	Coronal	Mean change %
Nit	0.3689 + 0.04 a	1.1600 + 0.51 a	4.1022 + 0.51 a	1.8771 + 0.42 a
MTAP	5.0750 + 0.31 b	8.7206 + 0.29 b	13.7387 + 0.50 b	9.1781 + 0.88 b
Control	0 c	0 c	0 c	0 c

* Different letters indicate significant difference between the percentage of reduction between the groups in the same third.

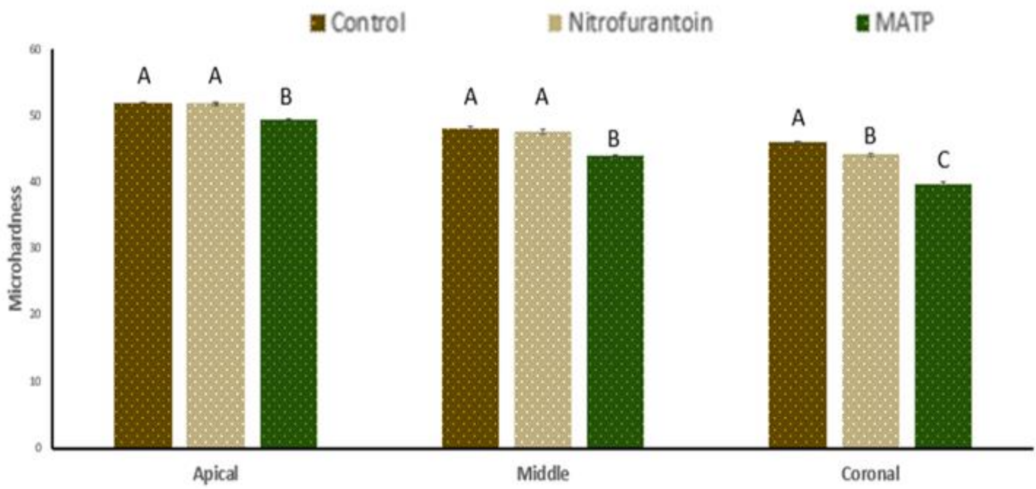


Figure 3.7 Microhardness value (HV) of the three root thirds (coronal, middle and apical) for the three groups, post-treatment (control, nitrofurantoin, and MTAP).

Different letters indicate significant difference between the same root third of the three groups.

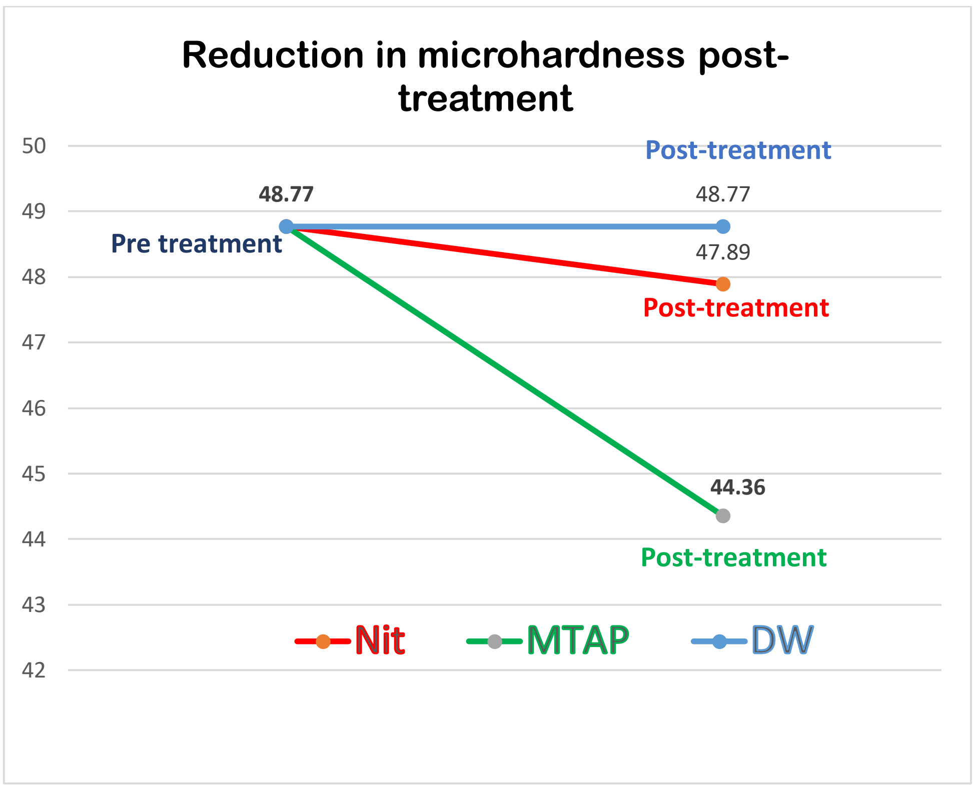


Figure 3.8 Reduction in microhardness value in post-treatment of the three different groups compared to the pre-treatment microhardness, in all of the three thirds of the root.

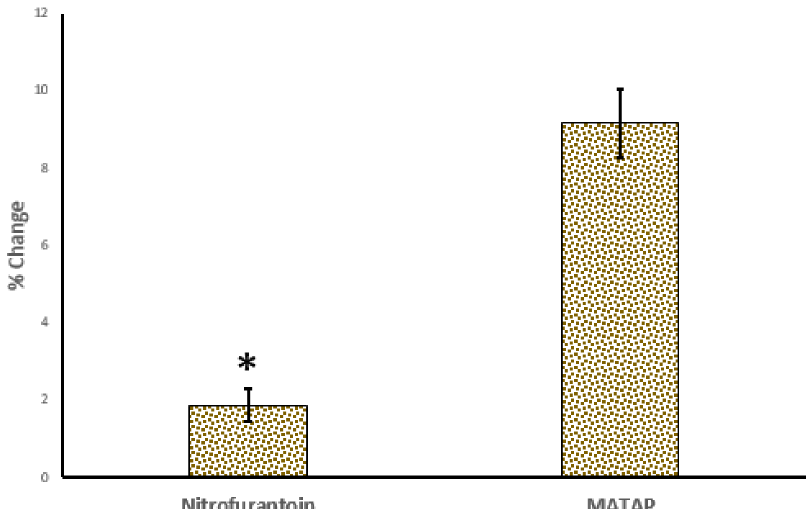
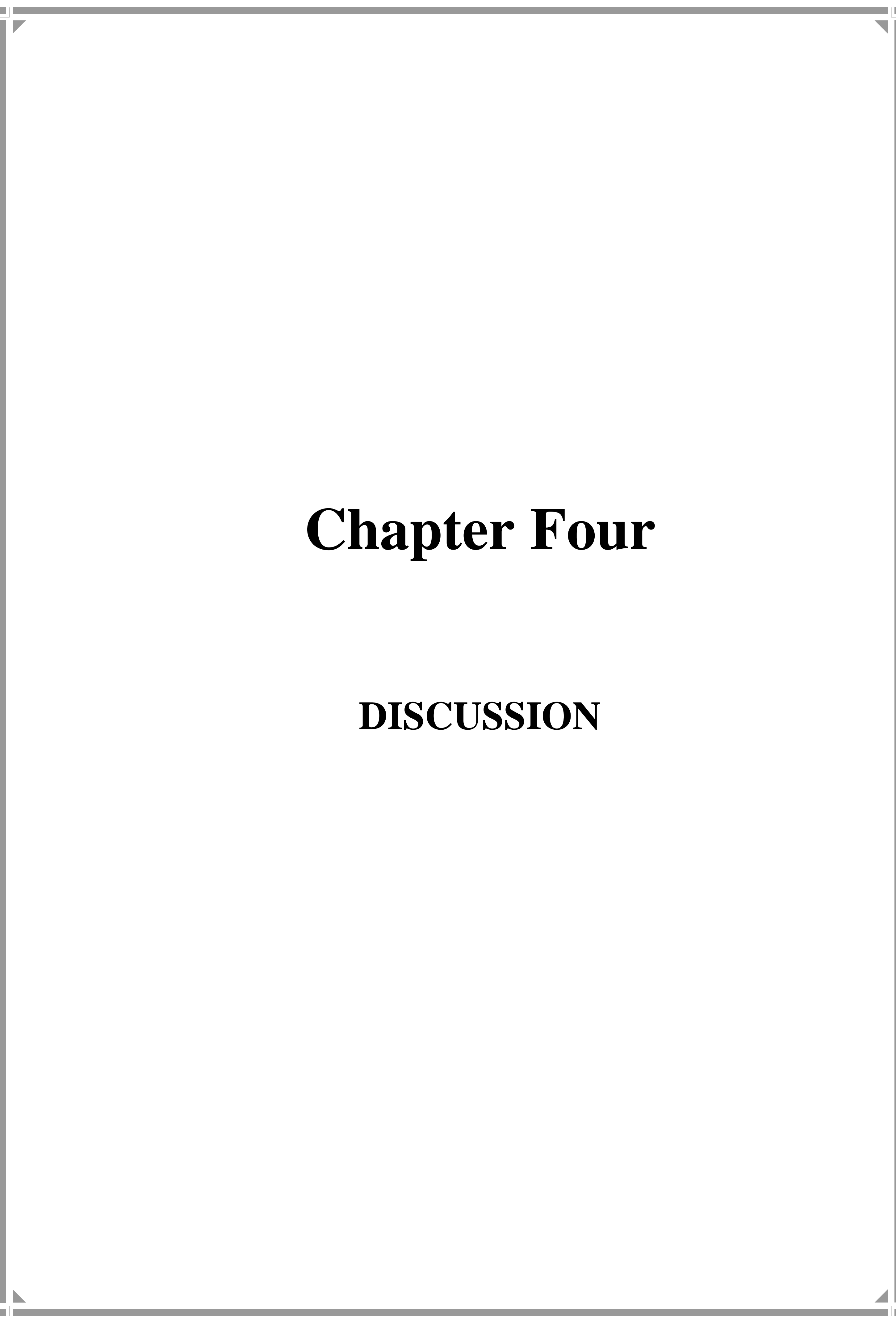


Figure 3.9 Mean percentage (%) of microhardness reduction of radicular dentin after application of nitrofurantoin and MTAP.

* $p<0.05$ compared to MTAP.



Chapter Four

DISCUSSION

Discussion

4.1 Assessment of the antimicrobial efficacy of nitrofurantoin and modified triple antibiotic mixture against *Enterococcus faecalis*. (In-*vitro* and ex-*vivo* study)

The leading cause of endodontic treatment failure is the persistence of microbial invasion of the root canal system and periradicular tissue (Lin *et al*. 1992). The infection of the root canal system is polymicrobial, containing both anaerobic and aerobic bacteria (Windley *et al*. 2005).

The treatment of a root canal is a procedure involving many steps like irrigation and mechanical instrumentation, which aim to make the root canals free of bacteria up to 50–70% (Peters *et al*. 2002; Nair *et al*. 2005). So the 30-50% of the root canal, which are not bacteria-free, will end in intracanal infection, and consequently periapical infection, leading to root canal treatment failure. That is why intracanal medicaments represent an additional step to achieve complete bacterial eradication, especially *Enterococcus faecalis* (Kawashima *et al*. 2009; Kumar *et al*. 2019).

Enterococcus faecalis, which is anaerobic, facultative, and gram-positive bacteria, is considered the most dominant causative microorganism resulting in persistent or secondary infection of root canals, as documented by culturing and molecular methods, leading eventually to failed root canal treatment. *Enterococcus faecalis* isolated from root canal failure cases owns several factors responsible for the high pathogenesis and persistence inside the root canal system (Stuart *et al*. 2006; Ozbek *et al*. 2009; Wang *et al*. 2012a).

Enterococcus faecalis produces extracellular proteases genes, like gelatinase and serine protease (gelE-sprE operon), which facilitates persistence through biofilm formation. Gelatinase will degrade the organic matrix in dentin, which has a significant predisposition in the infection of

the root canal system by *E. faecalis*. Furthermore, serine protease can break peptide bonds facilitating adherence of *E. faecalis* to dentin (Wang *et al*. 2011; Zoletti *et al*. 2011).

Additionally, there are other genes that help the adhesion of *E. faecalis* to the dentinal walls. One such a gene is the Enterococcal surface protein (ESP) gene, which accelerates the virulence, and increases colonization in the root canal system by production of biofilm. This biofilm helps *E. faecalis* to withstand the bactericidal effect of antimicrobials by reinforcing the bacteria to become 1000 times more resistant microorganisms to antimicrobial agents than the bacteria that cannot produce such biofilm (Toledo-Arana *et al*. 2001; Svensater and Bergenholtz 2004).

Meanwhile, collagen adhesion protein (Ace), antigen A (EfaA), and aggregation substance proteins (Agg) are genes increasing the adherence of *E. faecalis*. These adhesion factors will increase the colonization and adherence of *E. faecalis* to collagen Type I and extracellular matrix proteins found inside the dentin. Also, there is a gene called secretory metalloprotease gelatinase (gelE), which is another factor responsible for biofilm production in *E. faecalis*, causing root canal infection failure (Hubble *et al*. 2003).

Therefore, *E. faecalis* has been selected for this study, as it is the primary and most dominant microorganism found in failed root canal treatment. They have got importance because of their resistance to multiple antimicrobials (Sood *et al*. 2008). The three isolates were taken in order to evaluate more than one isolate of *E. faecalis* and to have some diversity, also to assess the possibility of antibiotic resistance that may evolve due to different genes in different isolates. Although the resistance characteristics differ in essential ways, they can generally be categorized as intrinsic resistance, acquired resistance, and tolerance (Hollenbeck and Rice 2012).

Thus, because of the increasing evidence suggestive of resistance of the *E. faecalis* to the commonly used intracanal medicaments (Leonardo *et al*. 2006; Vasudeva *et al*. 2017; Sabarathinam *et al*. 2018; Yadav *et al*. 2018), a more significant effort is done to develop materials that can eliminate *E. faecalis* from the root canal system completely.

Nitrofurantoin (Nit) was selected in this study because it has a broad spectrum of antibacterial activity and is both bactericidal and bacteriostatic against microorganisms (Maddison *et al*. 2008). Nitrofurantoin is the drug of choice against *E. faecalis*, and it has been used for an extended period in urinary tract infections (UTI), chronic and recurrent infections caused by *E. faecalis* (Butt *et al*. 2004; Qiao *et al*. 2013; Gardiner *et al*. 2019). Furthermore, resistant species are rare (Zhanel *et al*. 2003; Meena *et al*. 2017).

Nitrofurantoin is a unique antibiotic, owning a hydantoin ring with a nitro-substituted furanyl side chain, which will be metabolized by the bacteria to produce reactive compounds which have bactericidal action on the bacteria (Long *et al*. 2018). Unlike other antibacterial agents, nitrofurantoin has a unique mechanism of action, it will denature bacterial ribosomal proteins after being reduced by bacterial flavoproteins; this phenomenon will be repeated with other bacterial macromolecules. As a consequence, there will be suppression of many essential processes inside the bacteria like aerobic energy metabolism, cell wall synthesis, DNA synthesis, RNA synthesis and protein synthesis. Because of this enormous scope of suppression mechanisms, there is a very poor possibility of developing bacterial resistance to nitrofurantoin. Thus, bacterial resistance to nitrofurantoin is very rarely seen since its introduction and FDA approval in 1953 until now. It is very scarce to encounter cross-resistance with antibiotics or transferable resistance in bacteria (McOsker and Fitzpatrick 1994).

In this study, we have selected and used modified triple antibiotic paste (MTAP), which is a combination of three antibiotics; ciprofloxacin, clindamycin, and metronidazole, as a control group because we aimed to compare the antibiotic agent (nitrofurantoin), as an experimental intracanal medicament, with another intracanal medicament based on an antibiotic agent like (MTAP). Furthermore, to assess the efficacy of a single agent compared with a multi-drug paste; MTAP. Modified triple antibiotic paste is a modification of triple antibiotic paste (TAP) by replacing minocycline with clindamycin to prevent crown discoloration, as many studies found a severe color change occurred after one day of administration of TAP containing minocycline (Dodd *et al*. 1998; Lenherr *et al*. 2012; Kirchhoff *et al*. 2015; Jagdale *et al*. 2018).

Algarni *et al*. (2015) demonstrated that MTAP has similar efficacy as TAP against *E. faecalis* isolates. Mozayeni *et al* (2014), Ravi (2017), and Sabarathinam *et al*. (2018) showed that TAP resulted in better antibacterial efficacy, against *E. faecalis* than non-antibiotic-based intracanal medicaments such as chlorohexidine gluconate gel and calcium hydroxide, though it could not achieve complete elimination of *E. faecalis*, which if not completely eradicated during root canal treatment, *E. faecalis* will be transformed into a non-cultivable state and will survive the chemo-mechanical steps that are supposed to be bactericidal. Moreover, that bacteria have the capability to revert into a cultureable state when there is a suitable environment (Halkai *et al*. 2012). That is why it is necessary to find a medicament that can eliminate *E. faecalis* completely.

To achieve MBC concentration, an evaluation of the MIC should be performed. In our study, five sequential concentrations of nitrofurantoin and modified triple antibiotic mixture were used, including; 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, and 6.25mg/mL (as obtained from the serial dilution method in the current study). Usually, any MBC is equal to

or above the MIC value (Kiem and Schentag 2006; Sykes and Rankin 2014), in this study, all the MBC results were equal to the MIC results for each of nitrofurantoin and modified triple antibiotic mixture. The result of the current study showed that nitrofurantoin could eradicate all the isolates (S1, S2, and S3) completely with MBC of (6.25, 12.5, and 25mg/mL), respectively. In addition, the result of this study showed that the three clinical isolates of *E. faecalis* (S1, S2, and S3) were sensitive (S) to nitrofurantoin because its MIC results were ≤ 32 mg/mL according to the CLSI. While MTAP showed complete eradication of the three isolates only at 25mg/mL. Hence, those concentrations were used in the ex-*vivo* assessment.

In the current study, ex-*vivo* assessment was carried out on extracted teeth (*E. faecalis* isolates were inoculated inside the root canal system of the extracted teeth), the colony forming unit (CFU) of *E. faecalis* in the canal lumen was counted pre and post application of Nit paste and MTAP as intracanal medicaments. Additionally, the CFU of *E. faecalis* was counted in the dentinal chips samples, to evaluate the antimicrobial efficacy of those antibiotic pastes against *E. faecalis* that can attached to the dentin wall. Regarding the evaluation of Nit paste and MTAP against the first isolate (S1), which was isolated from the blood in a patient with sepsis, Nit showed a complete eradication with zero CFU in the canal lumen as well as the dentinal chips at the lowest concentration; (6.25mg/mL) and onwards, while MTAP could not eradicate this isolate from the root canal lumen at the lower concentrations, neither in 6.25 nor at 12.5mg/mL, but it could achieve a complete eradication with zero CFU at 25mg/mL upwards. This may be explained by the fact that this isolate is isolated from blood in a patient with sepsis so may have no resistance to Nit but has low resistance to MTAP, also possibly due to the lack of many factors that can contribute to the high resistance of S2 and S3 that were

isolated from failed endodontic infections. This can be justified by the fact that this isolate demonstrated some resistance to MTAP, which needed a high concentration of MTAP to overcome its resistance, in contrast to the Nit which achieved full eradication even with the lowest one.

On the other hand, when we used Nit and MTAP against the second isolate of *E. faecalis* (S2), which was isolated from a failed endodontic treatment patient without exposure to antibiotics within the last three months. Nitrofurantoin paste exhibited complete eradication of this isolate with zero CFU in the canal lumen as well as the dentinal chips at 12.5mg/mL increasingly. Meanwhile, it could not eliminate this isolate completely with viable bacteria seen at 6.25mg/mL. Concerning MTAP, it could eradicate this isolate completely at the same concentration as that for the first stain which is 25mg/mL and upwards, while at 6.25 mg/mL it could reduce this isolate but not to the degree of complete eradication from the root canal lumen.

Pertaining the third isolate of *E. faecalis*, being isolated from a failed endodontic treatment patient with an antibiotic course for two weeks' duration with no response. Nitrofurantoin paste achieved total eradication in the canal lumen as well as the dentinal chips at 25mg/mL and increasingly. Whilst, CFU was seen at lower concentrations of 6.25 mg/mL and 12.5mg/mL. About MTAP, again 25mg/mL was the concentration needed to reach a CFU of zero count.

As perceived from these results, MTAP was noted to show complete eradication at the same concentration (25mg/mL) regardless of the source of the isolate. In contrast, nitrofurantoin paste could eradicate S1 and S2 with lower concentrations (6.25 mg/mL, 12.5mg/mL), respectively, while for S3, it was 25mg/mL. This could be explained by the fact that MTAP encountered some resistance from *E. faecalis* at lower concentrations (6.25 mg/mL and 12.5 mg/mL); therefore, it needed higher doses to overcome

the resistant bacteria. Since MTAP is a combination of three antibiotics; metronidazole, ciprofloxacin, and clindamycin, so the possibility of resistance of *E. faecalis* to one or more of those antibiotics will interfere with its antibacterial effect.

Duh *et al*. (2001) and Singh *et al*. (2002) found that *E. faecalis* was resistant to clindamycin. It is known that enterococci are intrinsically resistant to clindamycin, which is mediated by the product of the lsa gene. Furthermore, a study by Dubey and Padhy (2015) found that 42% of *E. faecalis* was constitutively resistant to clindamycin.

On the other hand, Das *et al*. (2006), found that there was a high resistance of *E. faecalis* isolates that cultured from UTI to ciprofloxacin and high susceptibility to nitrofurantoin. Gaetti-Jardim *et al*. (2010) evaluated the resistance to antibiotics of species of aerobes and facultative anaerobes isolated from the oral cavity; they found that *E. faecalis* was resistant to ciprofloxacin. Moreover, Lee (2013) showed that ciprofloxacin is no longer a recommended therapy for *E. faecalis* from complicated UTI, as (47%) of the 265 isolated *E. faecalis* isolates were resistant to ciprofloxacin, whereas, Akhter *et al*. (2014) found in their study that (76.19%) of *E. faecalis* was resistant to ciprofloxacin.

In another study, Rams *et al*. (2013) concluded that metronidazole and clindamycin revealed poor in vitro activity against *E. faecalis* isolated from human subgingival samples and would likely be ineffective therapeutic agents against these species in periodontal pockets. However, the clinical isolates were generally sensitive to ciprofloxacin (89.4% susceptible; 10.6% intermediate resistant).

Concerning nitrofurantoin, Zhanel *et al*. (2001) have shown that nitrofurantoin is active against all isolates of *E. faecalis* found in UTI, demonstrating that they were susceptible to nitrofurantoin. Butt *et al*. (2004) found that, for a period of three years, nitrofurantoin was an

effective antibacterial in-vitro agent and can be used for the treatment of enterococcus urinary tract infections, as they showed that one hundred and twenty-seven isolates of enterococci were susceptible (88%) to nitrofurantoin. Abdulla and Abdulla (2006) showed that nitrofurantoin was effective against *E. faecalis* (cultured from UTI) in (97.3%), while ciprofloxacin was effective in only (35.7%).

Rahbar *et al*. (2007) found that nitrofurantoin had the lowest resistance rate compared to other antibiotics like ciprofloxacin against *E. faecalis* (cultured from UTI) (97 % vs. 33.38%, respectively). Chayakul *et al*. (2007) proved that the most active drugs against *E. faecalis* were nitrofurantoin. In addition, Toner *et al*. (2016) found that *E. faecalis* had a sensitivity test 100% to nitrofurantoin.

Sorlózano-Puerto *et al*. (2017) demonstrated that for four years, *E. faecalis* had a sensitivity to nitrofurantoin ranging from 95% to 100%.

The scientific base behind this high susceptibility of *E. faecalis* towards nitrofurantoin can be explained by the fact that nitrofurantoin uses several mechanisms to succeed as an antimicrobial agent (Squadrito and Portal 2019). The mechanism of action of nitrofurantoin is unique and complex as it owns both bacteriostatic and bactericidal effects. It kills bacteria through damaging all of DNA, RNA, protein, and cell wall protein synthesis (Blass 2015).

Moreover, through inhibiting acetylcoenzyme A, nitrofurantoin can prevent bacterial carbohydrate metabolism. It is confirmed that nitrofurantoin is bacteriostatic at low concentrations (5-10 mg/mL), but it is bactericidal at higher concentrations (Ebadi 2008), this explains the results of Nit in the present study.

The rare possibility of development of bacterial resistance to nitrofurantoin can be attributed to the nitrofurantoin's wide range of

mechanisms of action, as it destroys several major processes that are essential and vital to the bacterial cell (Batabyal 2018). Thus, there is rarely any bacterial resistance. Bacterial resistance emerges as a result of natural selection and mutation. The mechanism involved in the development of bacterial resistance to an antimicrobial like ciprofloxacin, metronidazole or any other ordinary antibiotic, relies on only one mechanism of action that should be overcome. In contrary, nitrofurantoin, having multiple mechanisms of action, requires various mutations in bacterial genes responsible for developing resistance to at least the four mechanisms listed, and those mutations should all be present in a single organism to be resistant to nitrofurantoin. Such possibility of occurrence is really rare that it almost never occurs (Blass 2015).

To our knowledge, no study is available about the use of pure nitrofurantoin paste as a single intracanal medicament against *E. faecalis* inside the root canal system. Besides, we compared between the bacterial growth inside the root canal lumen, as well as in the dentinal chips (which were harvested from the dentinal wall) after application of the intracanal medicaments. In all of the groups, we found that the number of remaining bacteria (CFU) in the dentinal chips was more than the number of the remaining bacteria inside the root canal lumen.

In group N (nitrofurantoin paste), the difference between the CFU in dentinal chips and the CFU in the canal lumen was statistically significant when using (6.25mg/mL) against S2 and S3, but it was non-significant with (12.5mg/mL) against S3. In group M (MTAP), the difference between the CFU in dentinal chips and the CFU in the canal lumen was statistically significant when using (6.25mg/mL) and non-significant with (12.5, mg/mL) against (S1), while the difference was statistically non-significant for both concentrations against S2 and S3. This is justified by the fact that

E. faecalis form a biofilm, it colonizes the dentinal walls by adhering to the mineral part, probably through Lipoteichoic acids (LTA) and to the collagen through Aggregation Substance (AS) and other surface adhesins (Kayaoglu and Qrstavik 2004). Moreover, *E. faecalis* has the ability to penetrate the dentinal tubules deeply because of their small size, which is enough for the bacteria to efficiently penetrate the tubules and live within them, in addition to the fact that they can tolerate periods of starvation (Wang *et al*. 2011; Al-Nazhan *et al*. 2014).

Furthermore, Portenier *et al*. (2002) demonstrated that the dentine itself can sometimes antagonize the bactericidal activity of the medicament. Thus, higher concentrations of the medicaments in a thick paste-like consistency are needed to combat these inhibitory effects. This can explain why the higher concentrations of those medicaments used in our study (25 mg/mL) could eliminate *E. faecalis* in both canal lumen and dentinal chips.

Those results can be further perceived when comparing them to the results of the zone of inhibition, where for S3 and S2 of *E. faecalis*, at MBCs, the diameters of the zone of inhibition of nitrofurantoin was highly significant larger than that of modified triple antibiotic mixture and according to the CLSI, the three clinical isolates of *E. faecalis* (S1, S2, and S3) were sensitive (S) to nitrofurantoin in all concentrations because in this study the results of zone of inhibition diameter were ≥ 17mm, except the concentration of (0.791 mg/mL) against (S3) which showed intermediate (I) result because the zone of inhibition diameter was (14.83mm).

This can be explained by the high antibacterial efficacy of nitrofurantoin against the more resistant isolates of *E. faecalis* (S3 and S2) which are isolated from the failed root canal teeth. Whereas, the diameter of the zone of inhibition for nitrofurantoin was slightly smaller than that of modified triple antibiotic mixture against the first isolate (S1) which was isolated from blood of a sepsis patient, but statistically it was non-significant. This

result may be attributed to the high susceptibility of this isolate towards the high concentration (25 mg/mL) of modified triple antibiotic mixture in comparison with the low concentration of nitrofurantoin which was (6.25 mg/ mL). So the results of the ex-*vivo* study are in agreement with the results obtained from the zone of inhibition test done in our study. The zone of inhibition in the current study was performed by the agar diffusion test which is the most commonly used method for the determination of antimicrobial activity of an antibiotic, especially the new intracanal medicaments. Additionally, the agar diffusion method is helpful in measuring and comparing the in-vitro antibacterial activities of those medicaments (Nalawade *et al*. 2016).

The limitation in the present study is that we studied the antibacterial effects of nitrofurantoin only against *E. faecalis*, which is the principal constituent of the microorganisms involved in persistent endodontic infections. Also, it was compared with an antibiotic based medicament and did not involve other non-antibiotic intracanal medicaments like chlorohexidine gluconate gel or calcium hydroxide.

Further studies are needed to assess the efficacy of nitrofurantoin against other microorganisms found in polymicrobial infections, as it is well known that nitrofurantoin has antibacterial action against both; gram-positive and gram-negative bacteria. And\or combining nitrofurantoin with an antifungal agent to combat the possible *Candida Albicans* species in those infections. Furthermore, we recommend further studies comparing nitrofurantoin effects with other non-antibiotic based intracanal medicaments against *E. faecalis* and other microorganisms found in polymicrobial infections in root canal treatment failure.

4.2 Evaluation of the biocompatibility of nitrofurantoin paste and MTAP in rats' subcutaneous connective tissue:

All dental materials that are used in the oral cavity (direct contact) should be harmless to all oral tissues, gingiva, mucosa, pulp, and bone. Furthermore, they should be free of agents that could elicit sensitization or allergic response in a sensitized patient (Mangala *et al*. 2015). From a

biological point of view, the irritant potential of any dental material must be evaluated, because eventual toxic components may cause irritation, degeneration, or even necrosis of the tissues adjacent to the material (Garcia Lda *et al*. 2010).

The biocompatibility of materials used in root canal treatment has been tested using different methods. The most common being subcutaneous implants, bone implants, cytotoxicity assays, and genotoxicity assays (Hauman and Love 2003a). But, according to ISO-6876 and 10993-5 standards (Camps and About 2003), implantation in the subcutaneous connective tissues of experimental animals has been extensively used to evaluate the biocompatibility of endodontic materials. Since it provides a more complete and clinically relevant information on the tissue response, through evaluation of local reactions to the material and examination of many complex interactions between the biological system and the material. It is more relevant than in vitro cytotoxicity tests, which do not provide the full picture of how a tissue reacts to the material under in *vivo* conditions (Moharamzadeh *et al*. 2009; Andolfatto *et al*. 2012; Soaresa *et al*. 2015).

In *vivo* studies, the use of laboratory animals for implantation of materials supplies more information about the inflammatory and immune reaction developed to the test material (Garcia Lda *et al*. 2010). Using rats is providing safer treatment and relevant results over a short period of time

due to the accelerated metabolism of these animals (Garcia Lda *et al*. 2011; Bretas *et al*. 2017).

Many studies have evaluated material biocompatibility by using different implantation vehicles, such as polyethylene tubes, silicon tubes, dentin tubes, and Teflon tubes (Koçak *et al*. 2014). In our study, we used polyethylene tube which is recommended because it is not irritant to the tissues (Hauman and Love 2003b; Gomes *et al*. 2007; Mori *et al*. 2014).

In this study, four different experimental period intervals have been used to assess the intensity of inflammation. In accordance with the American Dental Association (1972) three days, seven days, fourteen days, and thirty days have been used to permit the observation of histological responses during short, medium and long term periods. When the intention is to observe the intensity of events related to acute inflammation, short experimental periods of a few days should be chosen (1-3). During this period, the immediate response to the presence of the material is assessed; while the intermediate response is observed after periods of 7, 15, and 30 days (demonstrating the development of the reaction to the materials) (Scarparo *et al*. 2009). Additionally, in our study we used an ordinal scoring system for tissue response evaluation, and the histological investigations to evaluate the inflammatory response adjacent to the materials (Aminozarbian *et al*. 2012; Mousavinasab *et al*. 2012).

In this study, we used the MBCs of nitrofurantoin and MTAP against *E. faecalis* isolates (S2 and S3) that clinically isolated from failed endodontic therapy, and could eradicate them completely in the ex-*vivo* assessment. Those concentrations were (12.5 mg/mL) and (25 mg/mL) for nitrofurantoin paste that were considered as testing groups (TG1 and TG2) respectively, and one concentration of MTAP which was (25 mg/mL).

Histological inflammatory response has been evaluated adjacent to the testing groups (TG1 and TG2) and the results were compared with the

empty tube group that was considered as a negative control group(NC) and MTAP as a positive control group (PC). Furthermore, the pH was measured in order to see whether we can correlate the pH level of the tested medicament with the inflammatory response of the host tissue.

The result of the current study showed that, after implantation of 25mg/mL MTAP in (PC group), the intensity of the inflammation was moderate to severe on day 3 interval, while that inflammatory response declined gradually on day 7, 14, and 30 intervals being mild to moderate. The deference was statistically highly significant at all the period intervals. This may be due to the properties of the three antibiotic agents in MTAP (clindamycin, ciprofloxacin, and metronidazole), which have the ability to generate viable fibroblasts (Ferreira *et al*. 2010; Vijayaraghavan *et al*. 2012). Meanwhile, the severity of inflammation against (PC) group containing MTAP was higher than the inflammatory response against the two tested groups of nitrofurantoin with a concentration of 12.5 mg/mL in (TG1) and 25mg/mL in (TG2) and a statistically highly significant deference was found between them at all four periods. This can be explained by the fact that nitrofurantoin is a single agent compared to the multidrug combination of the MTAP and a single agent elicit less inflammatory response than a mixture of medications. Lu (2015) demonstrated that the toxicities of drug combinations to humans are increased with the increase of drug numbers. Additionally, the acidic nature of those antibiotic agents in MTAP with a pH of (4.9), could cause irritation to the tissue component, while nitrofurantoin with a neutral pH of (7.1) is more compatible with the intracellular compartment of the tissues. Bonnans *et al*. (2006) and Bathoorn *et al*. (2011) showed that exposure of normal epithelium to an acid can cause moderate to severe injury and consequently a more intense inflammatory response. Moreover, it was found that nitrofurantoin has less effects on than mammalian cells

than the bacterial cells, because bacterial cells activate the drug faster than do mammalian cells. (Batabyal 2018).

In the testing groups, when using two concentrations of nitrofurantoin paste; (12.5 mg/mL and 25mg/mL), the severity of inflammation against the higher concentration of 25mg/mL in (TG2) was more than that of lower concentration of 12.5 mg/mL in (TG1). On day 3 and 7, the intensity of the inflammation of those two groups showed mild to moderate response but the difference was statistically non-significant. Then the intensity of the inflammation was noticed to be decreasing on day 14 and 30 intervals being mild to no inflammation reaction and the difference was statistically non-significant too between them. On the other hand, the TG1 showed less inflammatory markers compared to the TG2 in all of the periods and this is due to the difference in the concentration used, where TG1 with a concentration of 12.5mg/mL being less inflammatory stimulant compared to the higher concentration of TG2 with the concentration of 25mg/mL and this can be attributed to the fact that the cytotoxicity of the antibiotic agents is dose dependent (Ferreira *et al*. 2010).

Furthermore, those results in the TG1, TG2, and the PC groups on day three can be attributed to the surgical trauma, in addition to the presence of the foreign chemical materials inserted inside the tissue (Gomes-Filho *et al*. 2012; De Oliveira Gonzalez *et al*. 2016).

On the other hand, in negative control group (NC) with an empty tube, the inflammatory reaction observed on day three, ranged from mild to normal then the intensity of the inflammation declined to normal in the rest of the time intervals which is similar to the results of other studies that were used an empty tube as negative control (Yavari *et al*. 2009; Aminozarbian *et al*. 2012). Initial inflammatory response to empty tubes is probably due to the surgical trauma in addition to the empty foreign body tubes inserted inside the tissue (Zmener *et al*. 1990; Ferreira *et al*. 2010). Any material

may elicit a tissue response if implanted inside the body tissue. In general, the host tissue harboring an implant encounters a wound healing process composed of foreign body reactions, inflammation, and fibrous encapsulation. Disruption of normal tissue leads to a process of wound healing as it attempts to repair itself. However, wound healing normally passes through four phases: hemostasis, inflammation, proliferation, and remodeling. (De Oliveira Gonzalez *et al*. 2016)

On day 3 and 7, the difference in the inflammatory response between the two tested groups (TG1 and TG2) and the negative control group with an empty tube (NC) was statistically significant, while on day 14 and 30 the difference being non-significant. Meanwhile, the difference of inflammatory response between positive control group using MTAP in (PC) and the negative control group with an empty tube (NC) was highly significant in all of the period intervals.

Thus, based on histopathological examination of the specimens, congested vessels were observed in TG1 and TG2 groups (nitrofurantoin 12.5 and 25mg/mL), revealing the inflammatory status of the specimens. It should be noticed that the nitrofurantoin displayed a tendency toward chronic inflammation, then the inflammation reaction decreased to mild-normal (no-inflammation), therefore indicating biocompatibility. since any material considered to be biocompatible if it doesn’t promote an inflammatory reaction and should be reduced to normal or non-significant reaction with time (Hauman and Love 2003a; Mori *et al*. 2009; Silveira *et al*. 2011; Mangala *et al*. 2015).

Therefore, according to the results of our study, it was found that nitrofurantoin at a concentration of 12.5mg/mL and 25mg/ml were biocompatible with the rat subcutaneous connective tissue and were comparable with the concentration of 25 mg/mL MTAP.

4.3 Evaluation the microhardness of the radicular dentin after using nitrofurantoin paste and MTAP.

Any chemical agent used as an intracanal medicament may negatively affect the chemical, physical, and mechanical properties of radicular dentine, therefore, it is mandatory to assess the effect of that intracanal medicament on the microhardness of the radicular dentin (Yassen *et al*. 2015; Elgendy and Nagy 2015; Krishan *et al*. 2017).

The correlation between microhardness and mineral structure of teeth has been found to be directly proportional. Angker *et al*. (2004) showed that the mechanical characteristics of dentin rely on its mineral content. Therefore, the measurement of microhardness can reflect the mineral loss or gain in dental hard tissue (Ballal *et al*. 2010; Massoud *et al*. 2017).

Alterations in the ratio of mineral composition might cause changes in the physical and mechanical properties of dentine (i.e. microhardness, solubility, and permeability) (Tuncer *et al*. 2015). Consequently, reduction in microhardness may increase the solubility and permeability of the root canal dentin which minimizes the sealing ability and adhesion of dental materials to dentin, resulting in suppression of resistance to bacterial invasion and facilitates coronal leakage (Poggio *et al*. 2012; Nikhil *et al*. 2016). Furthermore, reduction in microhardness may result in decreasing the modulus of elasticity and flexural strength of dentin leading to weakening of the root structure, consequently root canal-treated tooth is more apt to fracture (Saha *et al*. 2017).

Vickers microhardness is a measure of the resistance of material to deformation (Kandanuru *et al*. 2016). Vickers microhardness test represents an applicable and practical procedure to measure the surface changes of dental hard tissues after local application of chemical agents (Nikhil *et al*. 2016). It is more sensitive to measurement errors while it is

less sensitive to surface conditions, moreover, small specimens can be assessed precisely (Prabhakar *et al*. 2013; Eskandarinezhad *et al*. 2015).

The microhardness of dentine relies on the physical properties of the medicament used; pH and concentration, and on the structure of dentine; tubular density, location, and age (Zhang *et al*. 2010; Tuncer *et al*. 2015).

In this study, the microhardness value was measured from two indentations located at 500 µm from the canal lumen on the coronal side of each dentin third for standardization (Soram *et al*. 2016; Massoud *et al*. 2017). It was found that dentine microhardness depends on the location, the nearer the indentations to the pulp, the less the microhardness values (Prabhakar *et al*. 2013; Das *et al*. 2014).

Polishing of the coronal surface of the specimens was performed to remove any surface irregularities in order to have a mirror-like (glossy surface) to ensure the reflection of light, so that indentations can be accurately visualized during testing (Saha *et al*. 2017).

Since the microhardness of the radicular dentin changes at different locations within the same tooth (Ballal *et al*. 2010; Dineshkumar *et al*. 2012), indentations, in the current study, were made in the coronal, middle, and apical thirds of the radicular dentin for standardization. Those indentations were performed in the mesial and distal side of each third, since there is no difference in tubule density with the region (facial, lingual, mesial, or distal) (Komabayashi *et al*. 2008).

In this study we evaluated the microhardness before and after application of 25 mg/mL of nitrofurantoin paste, 25 mg/mL MTAP, and distilled water as a control group. The results showed a significant difference between pre-treatment versus post-treatment in the microhardness values (HV) in both nitrofurantoin paste and MTAP groups, but not in control group. The percentage of reduction in MTAP was

significantly greater than that of nitrofurantoin paste. This reduction in microhardness when using MTAP is attributed to the acidity of the antibiotic components of MTAP which was found in our study to be acidic (pH =4.9), and any acidic agent causes dentin demineralization (Adhani *et al*. 2015; Zhang *et al*. 2010; Tuncer *et al*. 2015; Parhizkar *et al*. 2018).

A study by (prather *et al*. 2014) showed a significant microhardness reductions in dentine when using MTAP in two concentrations (1mg/mL and 1g/mL) and they showed that the lower concentration (1mg/mL) with a pH of 6.6 caused less microhardness reduction than the higher concentration (1 g/mL) with a pH of 4.0 that caused strong demineralization of radicular dentine.

Furthermore, Krishan *et al*. (2017) showed a significant reduction in mean microhardness values when using (MTAP) at a concentration of (1 g/mL) compared to untreated dentin.

To date there are no reports on the effect of nitrofurantoin paste as an intracanal medicament on the microhardness of radicular dentine. The percentage of reduction in nitrofurantoin paste was significantly less than that of MTAP, which can be explained by the pH of nitrofurantoin which is neutral (7.1). Furthermore, nitrofurantoin paste has a single antibiotic agent in contrast to the MTAP which is composed of three antibiotics with 25mg of each agent in the mixture which increases the total quantitative concentrations of those antibiotics which will further affects the microhardness values of radicular dentine, since the pH and concentrations of the material used affect the microhardness (Zhang *et al*. 2010; Tuncer *et al*. 2015).

In this study, microhardness values was also evaluated in both pre-treatment and pot-treatment of the two medicaments in the three thirds of the root (coronal, middle, and apical thirds) and the results showed that

there were significant differences between the three thirds with microhardness values being the least in the coronal third, while those values were the highest in the apical third in both pre and post-treatment specimens. This can be explained by the fact that the microhardness of dentin depends on the tubular density which varies from an area to another, on the root dentin surface which decreases from cervical to apical dentin (Komabayashi *et al*. 2008). Furthermore, there is an inverse correlation between dentine microhardness and tubular density, as Pashley *et al*. (1985) stated that, when the tubular density increases, the dentin microhardness decreases. This is due to the reduction in the amount of calcified matrix between tubules as a result of an increase in the tubule density, which is also associated with a decrease in the amount of intertubular dentin.

Additionally, the histological pattern of the radicular dentin and relative nature of dentin in the apical region has an impact on the microhardness values. The apical portion of human teeth demonstrates various differences in structure, including accessory root canals, variable amounts of irregular secondary dentine, cementum-like tissue, low content of non-collagenous proteins and dentin sclerosis (Mjor *et al*. 2001).

Therefore, those histological variations in the root thirds have explained why MTAP and nitrofurantoin reduced the microhardness value at the coronal third more than the middle and apical third. But it is also worth noting that, at the apical and middle third of the root nitrofurantoin had little effect on the microhardness value of the radicular dentin with a non-significant different compared with distilled water in control group, unlike MTAP which showed a significant reduction. This may be attributed to the neutral pH of nitrofurantoin which is near to that of distilled water.

Chapter Five

CONCLUSIONS

AND

RECCOMENDATIONS

Conclusions and suggestions

5.1 Conclusions:

Within the limitations of this in vitro and ex-vivo study we concluded that:

1. Nitrofurantoin paste at a concentration of (25 mg/mL) is very potent as a single agent in achieving total eradication of clinical isolated of *Enterococcus faecalis* in endodontics.
2. Nitrofurantoin paste at concentrations of (12.5mg/mL) and (25 mg/mL) are biocompatible with the subcutaneous tissues of rat, and were comparable with that of MTAP (25mg/mL).
3. The use of 25mg/mL nitrofurantoin paste as intracanal medicament minimize the reduction in microhardness of radicular dentin, compared with the 25mg/mL MTAP.

5.2 Suggestions:

We suggested the following:

1. Assess the efficacy of nitrofurantoin paste against other minority microorganisms found in failed root canal treatment.
2. Combine nitrofurantoin paste with other antibiotics to widen its spectrum of antibacterial action to eradicate other isolated species, or combine nitrofurantoin paste with an antifungal agent to combat the possible *Candida albicans* species in those infections.
3. Further studies comparing nitrofurantoin paste effects with other used intracanal medicaments.
4. Study the effect of nitrofurantoin paste on the chemical structure of the radicular dentin.
5. Study the effect of nitrofurantoin paste on the physical properties of the radicular dentin (flexural strength, compressive strength…etc.).
6. Study the effect of nitrofurantoin paste on the push-out shear bond strength.
7. Study the effect of nitrofurantoin paste on the 3D seal of the obturating material.
8. Study the effect of nitrofurantoin paste in regenerative endodontics.
9. Study the color change of the crown when using nitrofurantoin paste.
10. Clinical study to evaluate the effect of the nitrofurantoin paste to minimize or relief the post-operative complications between visits (pain, tenderness, sensitivity…etc.).

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Ministry of Higher Education and
Scientific Research
University of Sulaimani
College of Medicine
Ethics committee

وەزارەتی خوێندنی باڵا و توێژینەوەی زانستی
سەرۆکایەتی زانکۆی سلێمانی
کۆلێجی پزیشکی
لیژنەی ئاکار

Number : 69
Date : 19 / 11 / 2019

The Ethics Committee of the College of Medicine

We the members of the ethical committee approved the research project below in the meeting (No : 3) on the date (19 / 11 / 2019).

Title of the research project:

Assessment of Nitrofurantion Antibiotic as an experimental intra canal medicament in Endodontics (An in vitro study).

Name and tile of the participants:

1- Lectura, DrMewan Salahaden AbdulRahman , B.D.S, M.S.c , PhD , Department of Conservative Dentistry College of Dentistry, University of Sulaimani.

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3- Prof. Dr.Kawa Fareq Dizaey, B.Sc.ph. M.Sc. ph.D, Department of Pharmacology College of Medicine, University of Hawler.

Place of research study: University of Sulaimani .

Members of Ethical committee of the College of Medicine

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Research Article

Assessment of Nitrofurantoin as an Experimental Intracanal Medicament in Endodontics

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Background and Objectives. Multiple antibacterial agents have been mixed and used as an intracanal medicament-like modified triple antibiotic paste (MTAP) to eliminate *Enterococcus faecalis* (*EF*), which has been most frequently identified in the cases of failed root canal treatment and periapical lesions. This study is aimed at using a single antibacterial agent, nitrofurantoin (Nit), as an experimental intracanal medicament paste against different clinical isolates of *EF* bacteria and at comparing its antimicrobial efficacy with MTAP. *Materials and Methods*. Three strains of *EF* (S1, S2, and S3) were clinically isolated. A total of 198 straight single-rooted human teeth were collected and divided randomly into three main groups: group N (Nit) ($n = 90$), group M (MTAP) ($n = 90$), and group W (distilled water) ($n = 18$). The main groups were subdivided into three subgroups according to the strain of *EF*: in groups N and M, subgroups S1, S2, and S3 ($n = 30$), while in group W, subgroups S1, S2, and S3 ($n = 6$). Then, each subgroup of N and M was divided into five groups ($n = 6$) according to the concentrations of Nit or MTAP (6.25, 12.5, 25, 50, and 100 mg/mL). The colony-forming unit (CFU) of *EF* from the canal lumen and dentinal chips was measured. *Results*. Nit could eradicate S1, S2, and S3 completely with concentrations of 6.25, 12.5, and 25 mg/mL, respectively, while MTAP showed complete eradication of the three strains only at 25 mg/mL. In all the groups, it was found that the CFU counts of *EF* in the dentinal chips were higher than those in the root canal lumen. *Conclusion*. At the concentration of 25 mg/mL, the Nit paste is effective in eradicating *EF* completely when it is used as an intracanal medicament.

1. Introduction

Several factors may cause a persistent periradicular infection as a consequence of root canal treatment like intraradicular infection, extraradicular infection, or foreign body reaction and cysts [1]. Those infections are the result of bacterial infection of the root canal, which will end in reinfection and failure of root canal treatment [2]. *Enterococcus faecalis* (*EF*), which is facultative bacteria, is the most predominant and most resistant microorganism leading to persistent periradicular lesions and eventually endodontic failure [3–5]. It is found in root canal failures in nearly 24–70% by culturing methods [6–8] and in 67–77% by molecular methods [9–12]. In other studies, it was retrieved as a major component, about 90% [13, 14]. This microorganism owns many special properties that enable it to survive in root canal and cause reinfection such as the ability to tolerate periods of starvation, deeply invade dentinal tubules [15], antimicrobial resistance, and the ability to adapt to changing environment [16].

Chemomechanical preparation of the root canal is considered the first step to reach the target of eradication of the intracanal bacterial invasion [17]. The chemomechanical procedures can reduce endodontic infection rather than ensure an immaculate root canal system; hence, microorganisms can survive inside the complex anatomy of the root canal system [18, 19]. Therefore, the intracanal medicaments represent an ideal reinforcement step to achieve the complete disinfection of the root canal system [20].

Local application of antibiotics to combat endodontic infections has been an option for years in endodontics, such as intracanal medicaments [21]. It represents a more successful route than systemic antibiotics to prevent the risks of

adverse side effects of antibiotics (like allergic reactions or toxicities) [22]. Local application of an antibacterial agent in the form of intracanal medicament gives the chance to target bacteria in each fine locus of the root canal system, which cannot be reached by conventional root canal treatment protocols such as instrumentation and irrigation [23]. Local application of antibiotics like intracanal medicament in endodontics enhances many positive prospects, including a complete or near-complete bacterial elimination, and higher local drug concentrations in addition to minimizing systemic adverse effects [24]. However, this technique may face a problem: the emergence of bacterial resistance [25, 26]. One of the main reasons leading to antibiotic resistance is the incorrect use of antibiotics, which will end in developing resistant microorganisms and, consequently, the formation of resistance genes and their inheritance from antibiotic-resistant to antibiotic-susceptible bacteria [27]. Because of the possibility of high tolerance and antibiotic resistance of *EF* to several antibacterial agents and antibiotics, any antibacterial agent has a short duration of action against *EF* until the development of resistance genes [28–30].

Triple antibiotic paste (TAP), which is a combination of three antibiotics, namely, metronidazole, ciprofloxacin, and minocycline, has been used as an intracanal medicament owing to its high antimicrobial effects [31]. There is a controversy between the studies supporting its efficacy to eradicate *EF* in the root canal system completely [32–35]. This may be due to emerging bacterial resistance [25, 26]. Another drawback of TAP is the crown discoloration due to its minocycline [36, 37]. Therefore, there has been a modification of TAP called modified triple antibiotic paste (MTAP) [38] by replacing minocycline with clindamycin. MTAP was shown to be as effective as TAP in reducing *EF* in the root canal system [39]. Due to those mentioned drawbacks of TAP and its modification to MTAP, there was a need for a new medicament that has less possibility of resistance, is equivalently potent against *EF*, and is preferably a single drug, so it needs less time and effort to prepare, is additionally cost-effective, and is a single drug rather than a multidrug.

Nitrofurantoin (Nit) is a synthetic nitrofuran compound [40]. It is effective against most gram-positive and gram-negative organisms [41]. It is a well-known antibacterial agent widely used as an oral antibiotic treatment for urinary tract infections (UTIs) [42]. Furthermore, it is the drug of choice for the treatment of infections caused by multidrug-resistant pathogens [43–45]. In a study that included 300 isolates of *Enterococcus*, none of the 300 isolates was resistant to Nit, including *EF* [46]. Several studies confirmed that Nit is highly effective against *EF* [47–49].

No previous studies have been done to explore the effect of Nit as a new intracanal medicament within the root canal system against *EF* in endodontics. Therefore, this study assessed the efficacy of Nit paste as an intracanal medicament in extracted teeth in comparison with MTAP.

2. Materials and Methods

2.1. Enterococcus faecalis Strain Source. Three strains of *EF* were used in this study, as shown in Figure 1. Strain 1 (S1) was taken from a blood sample of a sepsis patient, strain 2 (S2) was taken from a failed endodontic patient without antibiotics for the last three months, and strain 3 (S3) was taken from a failed endodontic patient on antibiotics.

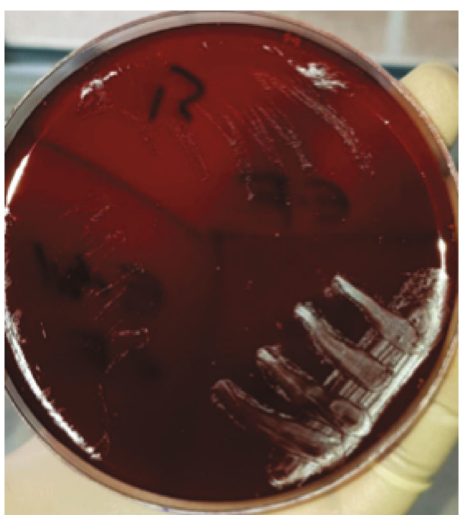

Figure 1: The isolated three strains of *Enterococcus faecalis*.

2.2. Patient Selection. Patients were interviewed and informed thoroughly about the study purpose, and informed written consent was signed before taking the samples of *EF*. The protocol of sampling was approved by the ethical committee at the College of Dentistry, University of Sulaimani.

The first strain S1 was isolated from a patient admitted for sepsis complaining of high fever, rigor, generalized aches and pains, tachycardia, sweating, leukocytosis, elevated ESR (Erythrocyte Sedimentation Rate), and CRP (Compliment Reactive Protein). Blood samples were taken and sent for blood culturing in the bacteriology department, and after 48 hours, *EF* was diagnosed as the causative factor of sepsis in this patient.

The second and third strains S2 and S3 were isolated from two patients complaining of failed endodontic treatment and requiring retreatment.

After obtaining a previous dental history, the patient's chief complaint was documented, and a clinical examination was performed and correlated with radiographic findings.

The two patients selected for the present study were in need of retreatment of their endodontically treated teeth. Each endodontic-treated tooth had a defective coronal seal with incomplete obturation of the root canal that was short filling which was more than 2 mm shorter than the radiographic root apex (radiographic presence of voids and radiolucent space running along some of the working length of the root filling) with a periapical radiolucency demonstrated in the periapical radiograph [50]. Additionally, the two teeth which had been selected for this study could be isolated with a rubber dam with no periodontal pockets more than 4 mm.

The patient from whom the S2 was isolated did not have local or systemic antibiotic administration within the last three months, while the patient who was the

source of S3 was on a course of antibiotics for two weeks with no response.

Exclusion criteria to select the two patients with failed endodontic treatment were smoking, pregnancy, diabetes mellitus, autoimmune disease, chemotherapy, immunosuppressive therapy, and malignancy.

2.3. Bacterial Sampling. Regarding the first strain (S1), the bacteria were isolated from the blood of the patient and then cultured on blood agar and incubated at 37°C for 48 hours and were examined by a bacteriologist and documented to be *EF*.

The second and third strains (S2 and S3) were isolated according to the procedure of root canal swabbing described by Gomes et al. [7] and Vineet et al. [50]. The selected tooth was isolated with a rubber dam, then it was disinfected with 5.25% sodium hypochlorite (Sultan Healthcare, Pennsylvania, USA); after that, it was inactivated with 5% sodium thiosulfate (The Science Company, Colorado, USA). The whole technique was under aseptic conditions. After removing the tooth filling, the root canal orifice was identified, followed by sterilization of the pulp chamber with 5.25% sodium hypochlorite; previous obturation was removed with Gates Glidden drills (Dentsply, Maillefer, Ballaigues, Switzerland) and endodontic files (Mani, Tochigi, Japan). Sterile saline was introduced inside the canal lumen to wet the canal. Then, two sterile paper points (Dentsply, Maillefer, Switzerland) were inserted into the full length of the canal and kept for 60 s. The paper points were placed into a 3 mL centrifuge tube containing 3 mL of reduced transport fluid (RTF) and transported to the microbiology department to perform the microbiological processing.

2.4. Laboratory Assessment. Three different strains were confirmed by the Phoenix and VITEK 2 system (DensiCHEK Plus, bioMérieux, Craponne, France) and by 99% identification with automated sensitivity reporting for all strains. The *EF* strains were cultured in brain heart infusion (BHI) broth (LAB M Limited/Neogen, Lancashire, UK) and incubated at 37°C for 48 h. To achieve a bacterial suspension with a concentration of 0.5 McFarland containing 1.5×10^8 cells/mL, the microbial cells were resuspended with saline [51].

2.5. Tooth Preparation. A total of 198 caries-free, straight single-rooted extracted human teeth were collected and stored in 0.9% physiological saline (B. Braun Medical Inc., Pennsylvania, USA) at room temperature until the time of use [52]. (The collection of the extracted teeth was done according to the study protocol approved by the Ethical Committee in the College of Dentistry, University of Sulaimani.) The crowns were cut perpendicularly to the long axis of the teeth from the cementoenamel junction (CEJ), with a rotary diamond disc (15LC diamond wafering blade, Buehler, Illinois, USA) in conjunction with physiological saline irrigation, and kept in 0.9% physiological saline. The root length was cut and standardized to 15 mm. After removing pulp tissue, canals were evaluated for apical patency and checked to have only one canal using #15 K-file (Mani, Tochigi, Japan) (roots with two canals were excluded from the study). The working length (WL) was determined by one mm short of the root apex, using a size 15 K-file, getting a 14 mm WL. The coronal third of the root canal was flared using Gates Glidden drills (#1, 2, and 3) [53], and the canals were instrumented within the WL with the Ni-Ti ProTaper rotary system (Dentsply, Maillefer, Ballaigues, Switzerland) (using sizes of S1, S2, F1, F2, and F3) at 3000 rpm (rotations per minute) speed and 2.5 N cm (Newton centimeter) torque with a micro motor handpiece (NSK-Nakanishi, Tokyo, Japan) (using S1 until 2/3rds of the working length, then Sx was used until the middle third; S1 was used again until the working length, after that using F1, followed by F2 and F3 for full working length) [54].

After each instrument change, 5 mL of 5.25% NaOCl was used for irrigation. Then, the samples were irrigated with 5 mL of 17% EDTA (ethylenediaminetetraacetic acid) (root canal preparation solution, Dline, Estonia, Europe) for smear layer removal. In order to achieve the effects of EDTA, a flush with 5.25% NaOCl for 5 min was done by using a special irrigation syringe. Then, each root was rinsed with 10 mL of physiological saline to remove the remnants of EDTA and NaOCl [55], using an endodontic irrigating syringe (Pacotech Inc., Texas, USA). Finally, the apical foramen of the root was sealed with a bonding agent and light-cured composite resin (Tetric N-Ceram, Ivoclar Vivadent, Liechtenstein) to prevent bacterial leakage. To prevent bacterial leakage from the accessory lateral canals, three layers of clear nail varnish (Orly International Inc., California, USA) were placed over all external root surfaces except for the coronal access and with care not to occlude the root canal entrance, and the teeth were allowed to dry [56].

2.6. Sterilization of Specimens. Each root specimen was placed in a sterile test tube containing 10 mL of brain heart infusion (BHI) broth; the tubes were placed in a large laboratory jar and autoclaved twice for 30 min at a temperature of 121°C and a pressure of 15 PSI (Zirbus Technology GmbH, Bad Grund (Harz), Germany) [57]. Then, those samples were kept in an incubator at 37° C for 24 hours (Memmert, Schwabach, Germany). Bacterial viability (contamination) and broth purity were checked.

2.7. Inoculation of Enterococcus faecalis Bacteria into the Specimens. All the steps of the bacteriology workup were done in a microbiological safety cabinet (Advancelab Pte. Ltd., Senang Cres, Singapore). All the samples (roots) were taken out of the BHI broth test tube aseptically by using a sterile tweezer. Then, the specimen was held by a sterile alcohol pad (70% isopropyl alcohol, Bolikim, China) to prevent contamination of the outer surface of the sample. The broth remaining inside the root canal was removed by aspiration using a sterile disposable syringe with a small gauge needle (BD, Franklin Lakes, New Jersey, USA).

The canals were inoculated with the suspensions of the three different strains of *EF* (S1, S2, and S3), with a standard concentration of 0.5 McFarland (1.5×10^8 CFU/mL), using a DensiCHEK devise (DensiCHEK Plus, bioMérieux, Craponne, France) to measure the optical density for each strain of *EF* suspension. Then, the sterilized canals were

filled with 20 μL inoculums of bacteria according to the strains by using a syringe with a sterile endodontic needle without spillage. Then, the orifice of the canal was closed by a sterile small cotton pellet (Kardelen Yazilim, Yenisehir, Turkey) and sealed with a temporary filling (TF) (Dline, Estonia, Europe).

The specimen (root) was wrapped by a wet sterile gauze (Nantong Jianan Medical Products Co. Ltd., Jiangsu, China) and was inserted in a new sterile test tube, and the cap was closed. The tubes were put in a sterile large laboratory jar and incubated for 21 days at 37°C [58].

2.8. Sample Grouping. The flowchart of the sample division is described in Figure 2. One hundred ninety-eight roots were divided blindly into three main groups:

(i) Group N (Nit) ($n = 90$) subdivided into three subgroups ($n = 30$), according to the strain of *EF* (S1, S2, and S3), then each subgroup was divided into 5 groups ($n = 6$) according to the MIC (minimum inhibitory concentration) of Nit used (6.25, 12.5, 25, 50, and100 mg/mL) (as measured in the pilot study)

(ii) Group M (MTAP) ($n = 90$) subdivided into three subgroups ($n = 30$), according to the strain of *EF* (S1, S2, and S3), then each subgroup was divided into 5 groups ($n = 6$) according to the MIC of MTAP used (6.25, 12.5, 25, 50, and 100 mg/mL)

(iii) Group W ($n = 18$) using DW as a negative control, then each group was subdivided into three subgroups ($n = 6$) according to the strain of *EF* used (S1, S2, and S3)

Bacterial viability was checked in three randomly selected tubes for each subgroup.

2.9. Preparation of Intracanal Medicaments. Four pure antibacterial powders were used: nitrofurantoin (Nit) (Procter & Gamble Company, Cincinnati, Ohio, USA), ciprofloxacin, metronidazole, and clindamycin (Skywalk Pharmacy, Wauwatosa, Wisconsin, USA).

To calculate the required amount of the antibacterial powder, an analytical balance was used (Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany). In this study, we prepared five concentrations of each medicament paste (Nit, MTAP); the concentrations are 6.25, 12.5, 25, 50, and100 mg/mL. To obtain a homogenous antibacterial paste, a magnetic stirrer (Cole-Parmer GmbH, Wertheim, Germany) was used for 2 hours at room temperature.

Group N (Nit paste): Nit solution was prepared by mixing pure powder of Nit with distilled water (DW) (AstraZeneca, Boston, Massachusetts, USA). Methylcellulose (MC) powder (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) was added to the Nit solution to get a thick paste-like consistency mixture [59]. 100 mg (Nit) + 1 mL (DW) + 80 mg (MC) were mixed to prepare 100 mg/mL Nit paste, while to prepare 50 mg/mL Nit paste, 50 mg (Nit) + 1 mL (DW) + 80 mg (MC) were mixed, and so on, for the other concentrations.

Group M (MTAP): MTAP was prepared by mixing equal proportions of pure powder of metronidazole, ciprofloxacin, and clindamycin with DW to prepare the MTAP solution. MC powder was added to this MTAP solution to get a thick, paste-like consistency. 100 mg (metronidazole) + 100 mg (ciprofloxacin)+100 mg (clindamycin)+ 1 mL (DW) + 80 mg (MC) were mixed to prepare 100 mg/mL MTAP paste, while to prepare 50 mg/mL MTAP, 50 mg (metronidazole) + 50 mg (ciprofloxacin) + 50 mg (clindamycin) + 1 mL(DW) + 80 mg (MC) were mixed, and so on, for the other concentrations.

Group W: 80 mg of MC was added to 1 mL of DW.

2.10. Application of the Medicament. After 21 days of incubation, the contaminated roots were taken out of the incubator. Each root was removed from the test tube, and the gauze was unwrapped. The specimen was cleaned with an alcohol pad. The TF and the cotton pellet were removed, and the canal content was aspirated. The aspirated content was cultured on blood agar (Oxoid Limited, Hampshire, UK) for evaluation of bacterial viability and measurement of CFU, then the root canal was irrigated with 5 mL DW to remove the bacterial suspension, and the canal was dried using three paper points. The Nit and MTAP paste were prepared, as mentioned before.

Each prepared medicament was injected into root canals by using size 27-gauge angled needles until the canal was filled with the medicament paste, as shown in Figures 3 and 4. The roots of the negative control group were injected with DW paste in the same way the medicament was injected. A sterile cotton pellet covered the canal orifice and was sealed with a TF, and the root was wrapped again with sterile wet gauze and placed inside a new sterile test tube. The specimens were returned to the incubator and kept there for seven days at 37°C.

2.11. Sampling of the Root Canal Lumen Content. At the end of the seven days of incubation, the specimens were extracted from the test tube, the gauze was unwrapped, the TF and the cotton pellet were removed, and the specimen was held in a sterile alcohol pad. Intracanal medicaments were evacuated from canals by irrigation with 10 mL of DW by using a sterile syringe. Then, two paper points were inserted into the canals and kept for 60 seconds [60]. Then, those paper points were kept in TG (thioglycollate) broth (LAB M Limited/Neogen, Lancashire, UK) through sterile test tubes. They were then incubated at 37°C for 24 hours. Then, subculturing is performed on blood agar at 37°C for 48 h. Growing colonies were counted and recorded as colony-forming units (CFU). To count the colonies of bacteria, we used the classical counting technique in the colony counter, and the results were given as a number of CFU (colony-forming unit).

2.12. Sampling of the Dentinal Chips. After the above step, assessment of the extent of infection of the radicular dentin is done depending on dentinal chips, which were obtained by shaving the full length of the root canal using a sterile #40 K-file [61] (tip diameter 0.40 mm) [62]. The dentinal chips were transferred by placing the file (just

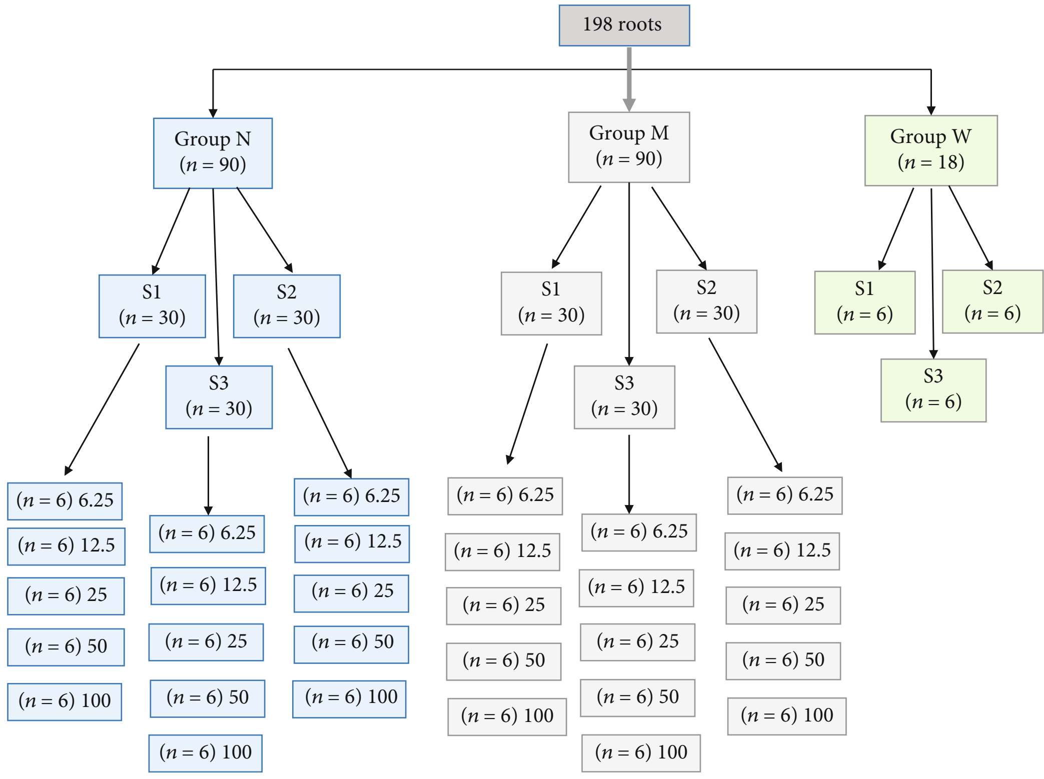

Figure 2: Flowchart showing the distribution of the roots among the groups.

Figure 3: Application of nitrofurantoin paste to the samples.

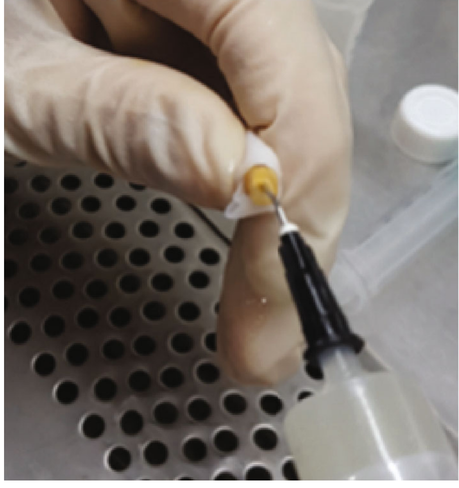

Figure 4: Application of MTAP to the samples.

its cutting surface) into TG (thioglycollate) broth (LAB M Limited/Neogen, Lancashire, UK) through sterile test tubes for 60 seconds (Figure 5). Then, they were incubated at 37°C for 24 hours. Then, subculturing is performed on the blood agar at 37°C for 48 h. Growing colonies were counted and recorded as colony-forming units (CFU).

2.13. Statistical Analysis. The results were evaluated statistically by using the Statistical Package for the Social Sciences (SPSS) version 23.0. All the data were expressed as mean ± SD. The Shapiro-Wilk test was used to determine normal distribution of the data. A Student t-test was used to compare the results. When the data was not normally distributed, the Mann-Whitney U test was used. Changes were considered statistically significant when the p value was 0.05 or less.

3. Results

3.1. Group N. The mean ± SD results of CFU of the three strains of *EF* of this group with different concentrations of Nit and the p value comparing CFU of the canal lumen and dentinal chips are shown in (Table 1). In this group, Nit

Figure 5: Incubation of dentin chips harvested from the samples.

was used at different concentrations (6.25, 12.5, 25, 50, and 100 mg/mL) against the three strains of *EF* (S1, S2, and S3) and the CFU was counted.

Strain S1: there was no CFU seen when using Nit at concentrations 6.25, 12.5, 25, 50, and 100 mg/mL.

Strain S2: there was no CFU found when using Nit at concentrations 12.5, 25, 50, and 100 mg/mL. When using Nit at a concentration of 6.25 mg/mL, CFU was found, and the CFU of *EF* from the canal (283.33 ± 47.72) was less than CFU of *EF* in dentinal chips (433.33 ± 33.33), and the difference was statistically significant ($p = 0.028$).

Strain S3: there was no CFU found when using Nit at concentrations 25, 50, and 100 mg/mL. When using Nit at a concentration of 6.25 mg/mL, CFU was noted, and the CFU of *EF* from the canal lumen (366.66 ± 49.44) was less than the CFU of *EF* in dentinal chips (516.66 ± 47.72), and the difference was statistically significant ($p = 0.05$). On the other hand, using Nit at a concentration of 12.5 mg/mL, CFU was seen, and the CFU of *EF* in the canal (266.66 ± 42.16) was less than CFU in dentinal chips (400.00 ± 63.24), and the difference was statistically not significant ($p = 0.11$).

3.2. Group M. The mean ± SD results of CFU of the three strains of *EF* of this group with different concentrations of MTAP and the p value comparing the CFU of the canal lumen and dentinal chips are shown in Table 2. In this group, MTAP was used at different concentrations (6.25, 12.5, 25, 50, and 100 mg/mL) against the three strains of *EF* (S1, S2, and S3) and the CFU was counted.

Strain S1: there was no CFU when using MTAP at concentrations 25, 50, and 100 mg/mL. When using MTAP at a lower concentration of 6.25 mg/mL, the CFU was recorded and the result of the CFU of *EF* in the canal lumen (183.33 ± 30.73) was less than the CFU of *EF* in dentinal chips (300.0 ± 25.81), and the difference was statistically significant ($p = 0.016$). Also using MTAP at a concentration of 12.5 mg/mL, the CFU was recorded, and the result of the CFU of *EF* in the canal lumen (66.66 ± 33.33) was less than the CFU of *EF* in dentinal chips (191.33 ± 60.09), and the difference was statistically not significant ($p = 0.120$).

Strain S2: there was no CFU when using MTAP at concentrations 25, 50, and 100 mg/mL. When using MTAP at a concentration of 6.25 mg/mL, the CFU was recorded and the result of the CFU of *EF* in the canal lumen (271.47 ± 54.26) was less than the CFU of *EF* in dentinal chips (416.66 ± 47.72), and the difference was statistically not significant ($p = 0.095$). Also using MTAP at a concentration of 12.5 mg/mL, the CFU was recorded, and the result of the CFU of *EF* in the canal lumen (200.00 ± 51.63) was less than the CFU of *EF* in dentinal chips (250.00 ± 56.27), and the difference was statistically not significant ($p = 0.52$).

Strain S3: there was no CFU when using MTAP at concentrations 25, 50, and 100 mg/mL. When using MTAP at a concentration of 6.25 mg/mL, the CFU was recorded and the result of the CFU of *EF* in the canal lumen (533.33 ± 42.16) was less than the CFU of *EF* in dentinal chips (683.33 ± 60.09), and the difference was statistically not significant ($p = 0.068$). Also using MTAP at a concentration of 12.5 mg/mL, the CFU was recorded, and the result of the CFU of *EF* in the canal lumen (333.33 ± 175.11) was less than the CFU of *EF* in dentinal chips (500.00 ± 57.73), and the difference was statistically not significant ($p = 0.1$).

3.3. Group W. The CFU was counted when using DW as a negative control. CFU was seen in all the samples of the three strains of *EF* (S1, S2, and S3). Likewise, the resulting CFU in the canal lumen was less than the CFU in dentinal chips, and the difference was statistically significant between them for the three strains, as shown in (Table 3).

Strain S1: there was CFU when using DW, and the result of the CFU of *EF* in the canal lumen (27000.0000 ± 3510.74381) was less than the CFU of *EF* in dentinal chips (39500.0000 ± 4847.3785), and the difference was statistically significant ($p = 0.01$).

Strain S2: there was CFU when using DW, and the result of the CFU of *EF* in the canal lumen (28283.3333 + 8584.80893) was less than the CFU of *EF* in dentinal chips (46500.0000 ± 5875.08865), and the difference was statistically significant ($p = 0.013$).

Strain S3: there was CFU when using DW, and the result of the CFU of *EF* in the canal lumen (57741.9165 + 4885.35226) was less than the CFU of *EF* in dentinal chips (64333.3333 + 6468.72819), and the difference was statistically significant ($p = 0.02$).

4. Discussion

The leading cause of endodontic treatment failure is the persistence of microbial invasion of the root canal system and periradicular tissue [63]. The infection of the root canal system is polymicrobial, containing both anaerobic and aerobic bacteria [64]. The treatment of a root canal is a procedure involving many steps like irrigation and mechanical instrumentation, which is aimed at making the root canals free of bacteria up to 50–70% [65, 66]. So the 30-50% of the root canal which are not bacteria-free will end in intracanal infection and, consequently, periapical infection, leading to root canal treatment failure. That is why intracanal medicaments

Table 1: The mean ± SD results of CFU of the three strains of *EF* when using Nit in different concentrations. The *p* value comparing CFU of the canal lumen and dentinal chips.

Strain of *EF*	Site of the sample of bacteria	Concentration of Nit (mg/mL) 6.25	*p* value	12.5	*p* value	25	50	100
S1	Canal lumen	0	—	0	—	0	0	0
	Dentinal chips	0		0		0	0	0
S2	Canal lumen	283.33 ± 47.72	0.028	0	—	0	0	0
	Dentinal chips	433.33 ± 33.33		0		0	0	0
S3	Canal lumen	366.66 ± 49.44	0.05	266.66 ± 42.16	0.11	0	0	0
	Dentinal chips	516.66 ± 47.72		400.00 ± 63.24		0	0	0

Table 2: The mean ± SD results of CFU of the three strains of *EF* when using MTAP in different concentrations. The *p* value comparing CFU of the canal lumen and dentinal chips.

Strain of *EF*	Site of sample of bacteria	Concentration of MTAP (mg/mL) 6.25	*p* value	12.5	*p* value	25	50	100
S1	Canal lumen	183.33 ± 30.73	0.016	66.66 ± 33.33	0.120	0	0	0
	Dentinal chips	300.0 ± 25.81		191.33 ± 60.09		0	0	0
S2	Canal lumen	271.47 ± 54.26	0.095	200.00 ± 51.63	0.52	0	0	0
	Dentinal chips	416.66 ± 47.72		250.00 ± 56.27		0	0	0
S3	Canal lumen	533.33 ± 42.16	0.068	333.33 ± 175.11	0.1	0	0	0
	Dentinal chips	683.33 ± 60.09		500.00 ± 57.73		0	0	0

Table 3: The mean ± SD results of CFU of the three strains of *EF* when using DW and the *p* value comparing the CFU of the canal lumen and the dentinal chips.

Strain of *EF*	Site of sample of bacteria	Mean ± SD	*p* value
S1	Canal lumen	27000.0000 ± 3510.74381	0.01
	Dentinal chips	39500.0000 ± 4847.3785	
S2	Canal lumen	28283.3333 ± 8584.80893	0.013
	Dentinal chips	46500.0000 ± 5875.08865	
S3	Canal lumen	57741.9165 + 4885.35226	0.02
	Dentinal chips	64333.3333 + 6468.72819	

represent an additional step to achieve complete bacterial eradication, especially *EF* [67, 68].

Enterococcus faecalis, which is anaerobic, facultative, and gram-positive bacteria, is considered the most dominant causative microorganism resulting in persistent or secondary infection of root canals, as documented by culturing and molecular methods, leading eventually to failed root canal treatment. *EF* isolated from root canal failure cases owns several factors responsible for the high pathogenesis and persistence inside the root canal system [5, 16, 69]. *EF* produces extracellular protease genes, like gelatinase and serine protease (gelE-sprE operon), which facilitate persistence through biofilm formation. Gelatinase will degrade the organic matrix in dentin, which has a significant predisposition in the infection of the root canal system by *EF*. Furthermore, serine protease can break peptide bonds facilitating adherence of *EF* to dentin [70, 71]. Additionally, there are other genes that help the adhesion of *EF* to the dentinal walls. One such a gene is the Enterococcal surface protein (ESP) gene, which accelerates the virulence, and increases colonization in the root canal system by production of biofilm. This biofilm helps *EF* to withstand the bactericidal effect of antimicrobials by reinforcing the bacteria to become 1000 times more resistant microorganisms to antimicrobial agents than the bacteria that cannot produce such biofilm [72, 73]. Meanwhile, collagen adhesion protein (Ace), antigen A (EfaA), and aggregation substance proteins (Agg) are genes increasing the adherence of *EF*. These adhesion factors will increase the colonization and adherence of *EF* to collagen type I and extracellular matrix proteins found inside the dentin. Also, there is a gene called gelE (secretory metalloprotease gelatinase E), which is another factor responsible for biofilm production in *EF*, causing root canal infection failure [74].

Therefore, *EF* has been selected for this study, as it is the primary and most dominant microorganism found in failed root canal treatment. They have importance because of their resistance to multiple antimicrobials [75]. The three strains were taken in order to evaluate more than one strain of *EF* and to have some diversity and also to assess the possibility of antibiotic resistance that may evolve due to different genes in different strains. Although the resistance characteristics differ in essential ways, they can generally be categorized as intrinsic resistance, acquired resistance, and tolerance [76]. Thus, because of the increasing evidence suggestive of resistance of the *EF* to the commonly used

intracanal medicaments [35, 77–79], a more significant effort is done to develop materials that can eliminate *EF* from the root canal system completely.

Nit was selected in this study because it has a broad spectrum of antibacterial activity and is both bactericidal and bacteriostatic against microorganisms [80]. Nit is the drug of choice against *EF*, and it has been used for an extended period in urinary tract infections and chronic and recurrent infections caused by *EF* [81]. Furthermore, resistant species are rare [82, 83]. Nitrofurantoin is a unique antibiotic, owning a hydantoin ring with a nitro-substituted furanyl side chain, which will be metabolized by the bacteria to produce reactive compounds which have bactericidal action on the bacteria [84]. Unlike other antibacterial agents, Nit has a unique mechanism of action. Nit will denature bacterial ribosomal proteins after being reduced by bacterial flavoproteins; this phenomenon will be repeated with other bacterial macromolecules. As a consequence, there will be suppression of many essential processes inside the bacteria like aerobic energy metabolism, cell wall synthesis, DNA synthesis, protein synthesis, and synthesis of RNA. Because of this enormous scope of suppression mechanisms, there is a very poor possibility of developing bacterial resistance to Nit. Thus, bacterial resistance to Nit is very rarely seen since its introduction and FDA approval in 1953 until now. It is very scarce to encounter cross-resistance with antibiotics or transferable resistance in bacteria [85].

We have used MTAP (which is a combination of three antibiotics: ciprofloxacin, clindamycin, and metronidazole) as a control group because we aimed to compare an antibiotic agent (Nit), as an experimental intracanal medicament, with another intracanal medicament (based on an antibiotic agent), furthermore, to assess the efficacy of a single agent compared with a multidrug paste, MTAP, which is a modification of TAP by replacing minocycline with clindamycin to prevent crown discoloration. A severe color change occurred after one day of administration of TAP containing minocycline [86, 87]. Algarni et al. [39] demonstrated that MTAP has a similar efficacy as TAP against *EF* strains. Several studies by Mozayeni et al. [32], Ravi [33], and Sabarathinam et al. [35] showed that TAP resulted in better antibacterial efficacy, against *EF* than nonantibiotic-based intracanal medicaments such as chlorohexidine gel and calcium hydroxide, though it could not achieve complete elimination of *EF*. If not completely eradicated during root canal treatment, *EF* will be transformed into a noncultivable state and will survive the chemomechanical steps that are supposed to be bactericidal. Moreover, that bacteria have the capability to revert into a culturable state when there is a suitable environment [88]. That is why it is necessary to find a medicament that can eliminate *EF* completely.

Any antibiotic has a minimum concentration to kill the bacteria and eradicate it completely called MBC (minimum bactericidal concentration). To achieve this critical concentration, an evaluation of the MIC (minimum inhibitory concentration) should be performed. Therefore, in our study, we used five sequential concentrations of Nit and MTAP including 100, 50, 25, 12.5, and 6.25 mg/mL (as obtained from the serial dilution method that was done in the pilot study). Any concentration resulting in a zero CFU was considered as the MBC.

As a result of this study, regarding the evaluation of Nit and MTAP against the first strain (S1), which was isolated from the blood in a patient with sepsis, Nit showed a complete eradication with zero CFU in the canal lumen as well as the dentinal chips, from the lowest concentration (6.25 mg/mL) onwards, while MTAP could not eradicate this strain from the root canal lumen at the lower concentrations, neither in 6.25 nor at 12.5 mg/mL, but it could achieve a complete eradication with zero CFU at 25 mg/mL upwards. This may be explained by the fact that this strain is isolated from blood in a patient with sepsis so it may have no resistance to Nit but has low resistance to MTAP and also possibly due to the lack of many factors that can contribute to the high resistance of S2 and S3 that were isolated from failed endodontic infections. This can be justified by the fact that this strain demonstrated some resistance to MTAP, which needed a high concentration of MTAP to overcome its resistance, in contrast to the Nit which achieved full eradication even with the lowest one.

On the other hand, when we used Nit and MTAP against the second strain of *EF* (S2), which is isolated from a failed endodontic treatment patient without exposure to antibiotics within the last three months, Nit exhibited complete eradication of this strain with zero CFU in the canal lumen as well as in the dentinal chips at 12.5 mg/mL upwards. Meanwhile, at 6.25 mg/mL, it could not eliminate this strain completely with CFU still seen at the given concentration. Concerning MTAP, it could eradicate this strain completely at the same concentration as that for the first stain which is 25 mg/mL, and it can reduce this strain but not to the degree of complete eradication from the root canal lumen at 6.25 upwards.

Pertaining the third strain of *EF*, being isolated from a failed endodontic treatment patient with an antibiotic course for two weeks' duration with no response, Nit achieved total eradication in the canal lumen as well as the dentinal chips at 25 mg/mL and above, whilst CFU was seen at lower concentrations of 6.25 and 12.5 mg/mL. With regard to MTAP, again, 25 mg/mL was the concentration needed to reach a CFU of zero count.

As perceived from these results, MTAP was noted to show complete eradication at the same concentration (25 mg/mL) regardless of the source of the strain. In contrast, Nit could eradicate S1 and S2 with lower concentrations (6.25 and 12.5 mg/mL, respectively), while for S3, it was 25 mg/mL. This could be explained by the fact that MTAP encountered some resistance from *EF* at lower concentrations (6.25 and 12.5 mg/mL); therefore, it needed higher doses to overcome the resistant bacteria. Since MTAP is a combination of three antibiotics, metronidazole, ciprofloxacin, and clindamycin, the possibility of resistance of *EF* to one or more of those antibiotics will interfere with its antibacterial effect. Duh et al. [89] and Singh et al. [90] found that *EF* was resistant to clindamycin. It is known that enterococci are intrinsically resistant to clindamycin, which is mediated by the product of the lsa gene, although the mechanism remains poorly defined [91]. Furthermore, a study by Dubey and Padhy [92] found that 42% of *EF* was constitutively resistant to clindamycin.

On the other hand, Das et al. [93] found that there was a high resistance of *EF* strains (cultured from UTI) to ciprofloxacin and high susceptibility to Nit. Chayakul et al. [94] showed that the most active drugs against *EF* were Nit. In another study, Gaetti-Jardim et al. [95] evaluated the resistance to antibiotics of species of aerobes and facultative anaerobes isolated from the oral cavity; they found that *EF* was resistant to ciprofloxacin. Rams et al. [96] concluded that metronidazole and clindamycin revealed poor in vitro activity against *EF* isolated from human subgingival samples and would likely be ineffective therapeutic agents against these species in periodontal pockets. However, the clinical isolates were generally sensitive to ciprofloxacin (89.4% susceptible, 10.6% intermediate resistant). Moreover, Lee [97] showed that ciprofloxacin is no longer a recommended therapy for *EF* from complicated UTI, as 47% of the 265 isolated *EF* strains were resistant to ciprofloxacin, whereas Akhter et al. [98] found in their study that 76.19% of *EF* was resistant to ciprofloxacin.

Concerning Nit, Zhanel et al. [46] have shown that Nit is active against all isolates of *EF* found in UTI, demonstrating that they were susceptible to Nit. Butt et al. [81] found that, for a period of three years, Nit was an effective antibacterial in vitro agent and can be used for the treatment of enterococcus urinary tract infections, as they showed that one hundred and twenty-seven (88%) isolates of enterococci were susceptible to Nit. Abdulla and Abdulla [47] showed that Nit was effective against *EF* (cultured from UTI) in 97.3%, while ciprofloxacin was effective in only 35.7%. Rahbar et al. [48] found that Nit had the lowest resistance rate compared to other antibiotics like ciprofloxacin against *EF* (cultured from UTI) (97% vs. 33.38%, respectively). Toner et al. [49] found that *EF* had a sensitivity test 100% to Nit. Sorlozano-Puerto et al. [99] demonstrated that for four years, *EF* had a sensitivity to Nit ranging from 95% to 100%.

To our knowledge, no study is available about the use of pure Nit paste as a single intracanal medicament against *EF* inside the root canal system. Besides, we compared the bacterial growth between inside the root canal lumen and in the dentinal chips after application of the intracanal medicaments. In all of the groups, we found that the number of remaining bacteria (CFU) in the dentinal chips was more than the number of the remaining bacteria inside the root canal lumen.

In group N, the difference between the CFU in dentinal chips and the CFU in the canal lumen was statistically significant when using 6.25 mg/mL against S2 and S3, but it was nonsignificant with 12.5 mg/mL against S3. In group M, the difference between the CFU in dentinal chips and the CFU in the canal lumen was statistically significant when using 6.25 mg/mL and nonsignificant with 12.5 mg/mL against S1, while the difference was statistically nonsignificant for both concentrations against S2 and S3. This is justified by the fact that *EF* colonizes the dentinal walls adhering to the mineral part, probably through LTA (lipoteichoic acids), and to the collagen through AS (aggregation substance) and other surface adhesins [100]; moreover, it has the ability to penetrate the dentinal tubules deeply because of their small size, which is enough for the bacteria to efficiently penetrate the tubules and live within them, in addition to the fact that they can tolerate periods of starvation [71, 101]. Furthermore, Portenier et al. [102] demonstrated that the dentin itself can sometimes antagonize the bactericidal activity of the medicament. Thus, higher concentrations of the medicaments in a thick paste-like consistency are needed to combat these inhibitory effects. This can explain why the higher concentrations of those medicaments used in our study (25 mg/mL) could eliminate *EF* in both dentinal chips and inside the canal system.

The limitation in the present study is that we studied the antibacterial effects of Nit only against *EF*, which is the principal constituent of the microorganisms involved in persistent endodontic infections. Also, it was compared with an antibiotic-based medicament and did not involve other nonantibiotic intracanal medicaments like chlorohexidine.

Further studies are needed to assess the efficacy of Nit against other microorganisms found in polymicrobial infections as it is well known to have antibacterial action against both gram-positive and gram-negative bacteria; combining Nit with an antifungal agent to combat the possible *Candida albicans* species in those infections also warrants further study. Furthermore, we recommend further studies comparing Nit effects with other nonantibiotic-based intracanal medicaments against *EF* and other microorganisms found in polymicrobial infections in root canal treatment failure.

5. Conclusions

At a concentration of 25 mg/mL, Nit paste is effective in eradicating *EF* completely when it is used as an intracanal medicament.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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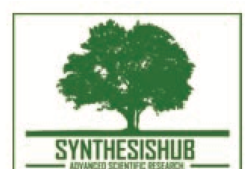

تقييم النايتروفيورانتوين كدواء تجريبي داخل قنوات جذور الأسنان في معالجة قنوات جذورالأسنان

دراسة مقدمة إلى مجلس كلية طب الأسنان ، جامعة السليمانية
كجزء من متطلبات درجة الدكتوراه في أختصاص معالجة الأسنان

من قبل

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٢٠١٩ م   ٢٧١٩ك   ١٤٤١هـ

الخلاصة:

الخلفية والأهداف: للقضاء على المكورات المعوية البرازية (*Enterococcus faecalis*) وهي البكتيريا الأكثر شيوعاً في حشوات قناة الجذر الفاشلة ، ولهذا الغرض تتم عملية تركيب عدة مضادات للبكتريا وإستخدامها كدواء داخل قنوات الجذور ، مثل معجون المضادات الحيوية الثلاثية المعدل والمسمى ب (MTAP) ، ومع ذلك ، بسبب العديد من سلبياته ، هناك بحث مستمر عن مضادات جديدة. لذلك ، تهدف هذه الدراسة إلى تقييم مضاد للبكتريا احادي التركيب ، نايتروفيورانتوين (Nit) ، كدواء تجريبي داخل قنوات الجذور ولتقييم تأثيره كمضاد للبكتيريا ضد المكورات المعوية البرازية ، كذلك تقييم توافقه الحيوي في الأنسجة تحت الجلدية للجرذان ، وتأثير هذا الدواء التجريبي على الصلابة المجهرية الدقيقة للعاج الجذري للسن، ومقارنة تلك النتائج مع نتائج معجون المضادات الحيوية الثلاثية المعدل.

المواد والطرق: تم عزل ثلاث سلالات من المكورات المعوية البرازية (س١ ، س٢ ، س٣) سريريًا. تم تحديد الحد الأدنى للتركيز المثبط (MIC) و الحد الأدنى للتركيز القاتل للبكتريا (MBC)لكل من نايتروفيورانتوين وخليط المضادات الحيوية الثلاثية المعدل المتكون من (سيبروفلوكساسين ، كليندامايسين ، ميترونيدازول) باستخدام طريقة التخفيف التسلسلي. ثم تم تقييم فعالية تلك الادوية كمضادات للبكتريا ضد السلالات الثلاثة من المكورات المعوية البرازية باستخدام (طريقة أنتشار أجار). تم إعداد ما مجموعه ٣٦ طبق بتري وتقسيمها إلى مجموعتين رئيسيتين ؛ المجموعة N ؛ (Nit) (ع = ١٨) ، والمجموعة M ؛ خليط المضادات الحيوية الثلاثية المعدل (ع = ١٨). تم تقسيم كل مجموعة إلى ثلاث مجموعات فرعية (ع = ٦) وفقًا لسلالة المكورات المعوية البرازية ، ويحتوي كل طبق بتري على سبعة تراكيز من المضادات الحيوية المشمولة بالدراسة. تم قياس قطر دائرة منطقة التثبيط وسجل القياس بالملليمتر. لغرض تقييم فعالية معجون النايتروفيورانتوين كمضاد للبكتريا، أجريت دراسة شبه واقعية خارج الجسم ، تم جمع ما مجموعة ١٩٨ من الأسنان البشرية الأحادية الجذر المستقيم والتي تم قلعها سابقا وتم تقسيمها بشكل عشوائي إلى ثلاث مجموعات رئيسية ؛ المجموعة N (Nit) (ع = ٩٠) ، المجموعة M (MTAP) (ع = ٩٠) ، والمجموعة W (ماء مقطر) (ع = ١٨). تم تقسيم المجموعات الرئيسية إلى ثلاث مجموعات فرعية وفقًا لسلالة المكورات المعوية البرازية ؛ في المجموعة N و M ، المجموعة س١ ، س٢ ، س٣ (ع=٣٠) ؛ بينما في المجموعة W ، المجموعة س١ ، س٢ ، س٣ (ع=٦). ثم تم تقسيم كل مجموعة فرعية من N و M إلى خمس مجموعات (ع

= ٦) وفقًا لتركيز Nit أو MTAP (١٠٠ ، ٥٠، ٢٥، ١٢,٥، ٦,٢٥ ملغم \ مل). تم قياس وحدة تشكيل مستعمرة (CFU) من المكورات المعوية البرازية من تجويف القناة ورقائق العاج. لتقييم التوافق الحيوي ، تم تقسيم ٢٤ من إناث فئران ويستار إلى أربع مجموعات حسب الفترة التجريبية وهي ٣، ٧ ، ١٤ ، ٣٠ يومًا تجريبيًا. تمت تعبئة أنابيب البولي إيثلين المعقمة بـ ١٢,٥ ملغم \ مل من معجون Nit كمجموعة تجريبية ١ (TG1) و ٢٥ ملغم \ مل من معجون Nit كمجموعة تجريبية ٢ (TG2) و ٢٥ ملغم \ مل من MTAP كمجموعة مقارنة إيجابية (PC) وزرعها تحت الجلد. تم زرع أنابيب فارغة كمجموعة مقارنة سلبية (NC) . بعد الفترات التجريبية ، تم إجراء الموت الرحيم للحيوانات بجرعة زائدة من الدواء المخدر. تم إستئصال مواقع الزرع ، وتم تحضير العينات للفحص النسيجي الكيميائي. تم تصنيف كثافة الإستجابة الألتهابية وفقًا لدرجات محددة. تم قياس مستويات الرقم الهيدروجيني للنايتروفيورانتوين بتركيز(١٢,٥ ملغم \ مل و ٢٥ ملغم \ مل) و (٢٥ ملغم \ مل) من محلول المضادات الحيوية الثلاثية المعدل بإستخدام مقياس رقمي لقياس الرقم الهيدروجيني . لتقييم الصلابة المجهرية الدقيقة ، تمت عملية تنظيف وتجهيز قنوات ل ١٨ جذر لأنياب بشرية غير متسوسة وتم تقسيمها إلى ثلاث مجموعات ؛ ٢٥ ملغم \مل Nit ، ٢٥ ملغم \ مل MTAP ومجموعة المقارنة السلبية (الماء المقطر). تم تقطيع الجذور بصورة أفقية إلى ثلاثة أجزاء حلقية (التاجية والمتوسطة والقممية). تم قياس تأثير معجون Nit و MTAP على الصلابة المجهرية الدقيقة للعاج الجذري بإستخدام جهاز (Vickers Microhardness Tester) قبل وبعد العلاج.

النتائج: كانت نتائج MBC لـ Nit و MTAP مطابقة لنتائج MIC لجميع السلالات. كانت نتائج MBCلـ Nit هي (٦,٢٥، ١٢,٥، ٢٥ ملغم\مل) ل (س١، س٢، س٣) على التوالي ، بينما بالنسبة لـ MTAP ، كانت النتيجة (٢٥ ملغم\ مل) للسلالات الثلاثة. أظهرت (طريقة أنتشار أجار) أن قطر دائرة منطقة التثبيط كان يتناسب طردياً مع تركيز كل من الأدوية المستخدمة ، حيث كان الأكبر في س١ ، والأصغر في س٣. في الدراسة الشبه واقعية خارج الجسم ، أظهرت النتيجة أن معجون النايتروفيورانتوين تمكن من القضاء تماماً على السلالات الثلاث(س١، س٢، س٣) بتراكيز (٦,٢٥ ، ١٢,٥، ٢٥ ملغم\مل) ، على التوالي. بينما أظهر MTAP قضاءاً تامًا على السلالات الثلاثة فقط بتركيز (٢٥ ملغم \ مل). ، أظهرت هذه الدراسة أن تعداد ال CFU لـ *E. faecalis*في شرائح العاج كان أعلى من تلك الموجودة في تجويف قناة الجذر. كشفت نتيجة تقييم التوافق الحيوي أن شدة الاستجابة الألتهابية كانت أكبر في مجموعة المقارنة الإيجابية (PC) من

المجاميع التجريبية ١و٢ (TG1) و(TG2) في جميع الفترات الزمنية التجريبية. في المجموعات التجريبية ١و٢ ، كانت شدة الاستجابة الألتهابية أكثر في TG2 من TG1 في جميع الفترات الزمنية. كانت شدة الأستجابة الألتهابية في مجموعة المقارنة السلبية (NC) أقل من المجموعات الأخرى. وكان مستوى الرقم الهيدروجيني للنايتروفيورانتوين متعادلا لكلا التركيزين ؛ (١٢,٥ ملغم \ مل) و (٢٥ ملغم \ مل) والتي كانت (٧,٠) و (٧,١) على التوالي ، على عكس الرقم الهيدروجيني للمضادات الحيوية الثلاثية المعدلة (٢٥ ملغم \ مل) والتي كانت حامضية (٤,٩). فيما يتعلق بتقييم الصلابة المجهرية الدقيقة ، أظهرMTAP بتركيز(٢٥ ملغم \مل) إنخفاضا في الصلابة المجهرية الدقيقة للعاج الجذري للأسنان بنسبة مئوية(٩,١٨ ٪) وهي أكبر بكثير من تلك النسبة ل Nit بتركيز (٢٥ ملغم\ مل) حيث كانت (١,٨٨ ٪) بالمقارنة مع مجموعة المقارنة. يضاف الى ذلك ، فإن قيم الصلابة المجهرية الدقيقة كانت الأعلى في الثلث القممي وتنخفض القيم لتكون الأدنى في الثلث التاجي وكانت الأختلافات بين جميع المجموعات في جميع الأثلاث الثلاثة ذات دلالة إحصائية.

الاستنتاجات: وفقًا لنتائج الدراسة الحالية ، معجون النايتروفيورانتوين بتركيز (٢٥ ملغم\مل) ، تمكن من القضاء على المكورات المعوية البرازية تمامًا عند إستخدامه كدواء داخل قنوات الجذور للأسنان، و متوافق حيوياً مع النسيج الضام تحت الجلدي للجرذ، إضافتا الى أنه يؤثر بشكل أقل على إنخفاض الصلابة المجهرية الدقيقة لعاج الأسنان الجذري مقارنة بـ ٢٥ ملغم\مل من MTAP.

الكلمات ذات الصلة: المكورات المعوية البرازية ، دواء داخل قنوات جذور الأسنان، الصلابة المجهرية الدقيقة ، معجون المضادات الحيوية الثلاثية المعدل ، نايتروفيورانتوين.

هەڵسەنگاندنی دەرمانی دژەهەوکردن نایترۆفیورانتۆین (nitrofurantoin) وەک چارەسەری ئەزموونی ناو کەناڵی ڕەگی ددان

تێزێك پێشكەش كراوە بۆ ئەنجومەنی كۆلیژی پزیشكی ددان ,
زانكۆی سلێمانی وەك بەشێك لە داواكارێكانی بەدەست هێنانی
بڕوانامەی دكتۆرا لە زانستی چارەسەركردنی ددان

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پوختەی توێژینەوەکە:

پێشینە و ئامانجەکان: بە مەبەستی نەهێشتنی بەکتریای ئینتیرۆکۆکەس فیکالس (*Eenterococcus faecalis*) کەبەربلاوترین بەکتریایە تایبەت بە چارەسەرکردنی کەنالی ڕەگی ددان. چەند جۆرێك دژە بەکتریای جیاواز تێکەڵکراون بۆ ئەوەی وەك دەرمانی چارەسەرکردنی ناو کەنالی ڕەگی ددانەکان بەکار بهێندرێت, بۆ نمونە هەویری دژەبەکتریای سیانی ئامادەکراو (MTAP). هەرچەندە لەبەرهەندێك کەم و کورتی و ڕێگری گەڕان بەدوای چارەسەری تردا بەردەوامە. بۆیە ئەم توێژینەوەیە ئامانجی ئەوەیە کە دژەبەکتریای تاك دانەیی/ماددەی دژە هەوکردن (nitrofurantoin –Nit) هەڵسەنگێنێت وەك هەویریکی چارەسەرکردنی ناو کەنالی ڕەگی ددانەکان وە هەڵسەنگاندنی کاریگەریەکەی دژی بەکتریای ئینتیرۆکۆکەس فیکالس وە هەروەها گونجانی لەگەڵ شانەی ژێر پێستی جرجەکان وە کاریگەری لەسەر ڕەقی عاجی ڕەگی ددانەکان وە بەراوردکردنی دەرئەنجامەکان لەگەڵ هەویری دژەبەکتریای سیانی ئامادەکراو (MTAP).

ماددەکان و ڕێگاکانی جێبەجێکردنەکە: سێ شێوازی ئینتیرۆکۆکەس فیکالس (S1, S2, S3) بە شێوەی پزیشکی جیاکراونەتەوە. کەمترین خەستی کپکردنی بەکتریا (MIC) لە گەڵ کەمترین خەستی دژەبەکتریا (MBC) ی دژەبەکتریای تاك دانەیی (ماددەی دژە هەوکردن) وەر گیراوە لەگەڵ تێکەڵەی دژەبەکتریای سیانی ئامادەکراو کە ئەوانیش بریتین لە (ciprofloxacin, clindamycin, and metronidazole) لە ڕێگەی کەمکردنەوەی خەستیەکەیان یەك لەدوای یەك. پاشان توانای دژەبەکتریایی ئەو دەرمانانە هەڵسەنگێندرا لەگەڵ ئەو سێ شێوازەی ئینتیرۆکۆکەس فیکالس لە ڕێگەی تەنینە وەی سەر ئەگار. کۆی 36 قاپی پێتری تاقیگەیی ئامادەکرابوون و دابەش کران بۆ دوو گرووپی سەرەکی: گرووپی N, کە ئاماژەیە بە دژەبەکتریای تاك دانەیی (Nit n=18) وە گرووپی M کە ئاماژەیە بە (تێکەڵەی دژەبەکتریای سیانی ئامادەکراو-MTAP n=18) . وە هەرگرووپێکیش دووبارە دابەشکرابۆوە بۆ سێ گرووپی لاوەکی تر (n=6) . وە بە پێی سێ شێوازەکەی ئینتیرۆکۆکەس فیکالس هەر قاپێکی پێتری تاقیگەیی حەوت دژەبەکتریای خەستکراوەیی تێدابوو, وە زۆنی کپکردنەکە بە ملیمەتر پێوانەکراوە و تۆمارکراوە. بۆ هەڵسەنگاندنی توانای دژە بەکتریای هەویری تاك دانەیی (نایترۆفیورانتوین) لە ڕێگەی ئامێری پێوانکردنی تاقیگەیی (ex-vivo) وە کراوە, ئەوا کۆی 198 ددانی دەرهێنراوی مرۆڤ کە یەك ڕەگ بوون کۆکراونەتەوە و کوێرانە دابەشکراون بەسەر سێ گرووپی سەرەکی: گرووپی N, گرووپی کە ئاماژەیە بە (Nit n=90) وە گرووپی M کە ئاماژەیە بە (تێکەڵەی دژەبەکتریای سیانی ئامادەکراو MTAP- n=90) وە گرووپی W (ئاوی دڵۆپێزاوn=18) وە ئەو گرووپە سەرەکیانەش دووبارە دابەش کرابوون بۆ سێ گرووپی تر بە پێی شێوازی سیانیەکەی ئینتیرۆکۆکەس فیکالس, لە گرووپەکانی N و M دا شێوازەکانی (S1,S2,S3) یەکسان بوو بە n=30 لە کاتێکدا لە گرووپی W دا شێوازەکانی

(S1,S2,S3) یەکسان بوو بە n=6 . پاشان هەر گرووپێکی لاوەکی (N و M) دووبارە دابەشکراوەتەوە بۆ پێنج گرووپ واتا (n=6) بە پێی خەستی دژەبەکتریای تاك دانەیی (nitrofurantoin –Nit) یان هەویری دژەبەکتریای سیانی ئامادەکراو (6.25, 12.5, 25, 50, 100 mg/ml)بەشی دروستبوونی بەکتریا لە ئینتیرۆکۆکەس فیکالس, لە ناو کەناڵی ڕەگی ددان و نەرمە توێژاڵی عاجی ددان پێوانە کراوە. بە مەبەستی هەڵسەنگاندنی گونجانی بایۆلۆجیان, 24 جرجی مێینەی ویستار دابەشکران بۆ چوار گروپ تاقیکردنەوەی 3 ڕۆژی 7 ڕۆژی 14 ڕۆژی 30 ڕۆژی. تیوبە پاکژکراوەکانی پۆلیسلین بە 12.5 mg/mL پڕکران لە هەویری دژەبەکتریای تاك دانەیی (Nit) وەك گروپی تێستکراوی ژمارە یەك (TG1) دانران, وە 25 mg/mL لە هەویری دژەبەکتریای تاك دانەیی (Nit) وەك گروپی تێستکراوی ژمارە دوو (TG2) , وە 25 mg/mL لە هەویری دژەبەکتریای سیانی ئامادەکراو وەك گروپی کۆنترۆڵکردنی پۆزەتیڤ (PC)پاشان لەژێر پێستدا چێندران. وە تیوبی بەتاڵیش وەك گروپی کۆنترۆڵکردنی نێگەتیڤی (NC) دانران دوای تەواوبونی ماوەی تاقیکردنەوەکان, زیندەوەرەکان بەهۆی دەرمانی بەنجکردنی زیادەوە مردن وە شوێنی چاندنەکانیش بەتەواوی لابران, پاشان نمونەکان ئامادەکران بۆ لێکۆڵینەوەی کیمیایی. پۆلێنکردنی توندی پرۆسەی هەوکردنەکان بەپێی نمرەی دیاریکراو ئەنجامدران. وە ئاستەکانی (pH) ترشی (12.5 mg/mL, 25mg/mL) لە دژەبەکتریای تاك دانەیی/ماددەی دژەهەوکردن (nitrofurantoin) وە(25 mg/mL) لە چارەسەری دژەبەکتریای سیانی ئامادەکراو, پێوانە کران لە ڕێگەی بەکارهێنانی ئامێریکی دیجیتاڵی پێوانەکردنی (pH). بۆ هەڵسەنگاندنی ڕەقیەکەی , 18 ڕەگی ددانی خراپ نەبوو بەکارهێنرا و دابەشکرا بۆ سێ گروپ:25 mg/mL Nit, 25 mg/mL MTAP وە کۆنترۆڵی نێگەتیڤ (ئاوی دڵۆپێژاو) وە ڕەگەکانیش دابەشکران بۆ سێ بەشی یەکسان (coronal, middle, apical) کاریگەری هەر هەویرێك لەسەر ڕەقی عاجی ددانەکان پێوانەکرا لەڕێگەی بەکارهێنانی ئامێری تاقیکردنەوەی ڤیکەرز لە پێش و دوای چارەسەرکردن.

دەرئەنجامەکان: ئەنجامی کەمترین خەستی دژەبەکتریاکە (MBC) بۆ هەر یەکە لە دژەبەکتریای تاك دانەیی/ماددەی دژەهەوکردن لەگەڵ تێکەڵەی دژەبەکتریای سیانی ئامادەکراو هاوشێوەبوون لەگەڵ کەمترین خەستی کپکردنی بەکتریا (MIC) لە هەموو شێوازەکاندا. ئەنجامی کەمترین خەستی دژەبەکتریاکە (MBC) ی دژەبەکتریای تاك دانەیی/ماددەی دژەهەوکردن بۆ هەرسێ شێوازەکە (S1,S2,S3) بریتیبوو لە (6.25, 12.5, 25 mg/mL) بەڵام بۆ تێکەڵەی دژەبەکتریای سیانی ئامادەکراو بریتیبوو لە (25 mg/mL) بۆ هەر سێ شێوازەکە. ڕێگەی تەنینە وەی سەر ئەگار ئەوەی نیشاندا کە تیرەی زۆنی کپکردنەکە ڕاستەوخۆ هاوڕێژەبوون لەگەڵ خەستی هەردوو چارەسەرەکە کە لە S1 دا گەورەترین بوو وەلە S3 بچووکترین بوو. لە ئامێری پێوانکردنی تاقیگەیی (ex-vivo) ئاسایە, ئەنجامەکە ئەوەی نیشان دەدا کە هەویرێکی

دژەهەوەکردنی تاك دانەیی دەتوانێت لە هەر سێ شێوازەکەی ئینتیرۆکۆکەس فیکالس (S1,S2,S3) بەتەواوەتی لە ناو ببات لە هەر سێ چڕیەکانی (6.25, 12.5, 25 mg/mL) بەڵام تێکەڵەی دژەبەکتریای سیانی ئامادەکراو تەنها لە چڕی (25 mg/mL) دەتوانێت بە تەواوی لە ناو ببات. لە هەموو گروپەکاندا دەرکەوتووە کە ژمارەی دروستبوونی بەکتریای ئینتیرۆکۆکەس فیکالس لە نەرمە توێژاڵی عاجی ددانەکان بەرزترە لە ناو کەناڵی ڕەگی ددانەکان. هەر وە ئەنجامی هەڵسەنگاندنە بایۆلۆجیەکە ئەوەی دەرخستووە کە توندی کاردانەوەی هەوکردنەکە لە گروپی کۆنترۆڵکردنی پۆزەتیف (PC) زیاتربووە وەك لە گرووپە تێستکراوەکانی یەك و دوو (TG1 , TG2) لە ماوەی کاتە دیاریکراوەکاندا . لە گرووپە تێستکراوەکاندا توندی کاردانەوەی هەوکردنەکە لە گرووپی دووەمدا زیاتربوو وەك لە گرووپی یەکەم لەماوەی سە رجەم کاتی هەڵسەنگاندنەکان. وە توندی کاردانەوەی هەوکردنەکە لەگروپی کۆنترۆڵکردنی نێگەتیڤ (NC) کەمتربوو لە گرووپەکانی تر. ئاستی (pH) ترشی دژەبەکتریای تاك دانەیی/ماددەی دژەهەوکردن, مام ناوەند بوو بۆ هەردوو خەستیەکانی (12.5mg/mL and 25 mg/mL) کە(7.0 وە 7.1) بوون وەکو ئاستی ترشی دژەبەکتریای سیانی نەبوو کە بریتیبوو لە (4.9)واتا ترش بوو. سەبارەت بە هەڵسەنگاندنی ڕقیەکەی, تێکەڵەی دژەبەکتریای سیانی ئامادەکراو (25 mg/mL) بە ڕێژەیەکی بەرچاو ڕەقیەکەی کەمیکرد لە عاجی ددانەکەدا بە ڕێژەی لەسەدا 9.18 % بە بەراورد لەگەڵ دژەبەکتریای تاك دانەیی/ماددەی دژەهەوکردن (25 mg/mL) کە بە ڕێژەی لە سەدا 1.88 % بوو کاتێك بەراوورد دەکرێن لەگەڵ گرووپەکانی کۆنترۆڵکردندا. هەرچەندە بەهای ڕەقیەکەیان لە بەشی سێهەمی کۆتا کەناڵەکەدا بەرزترین بوو وە پاشان کەمیکرد و بوو بە کەمترین بەها لە بەشی سێهەمی سەرەتا کەناڵەکەدا وە جیاوازیەکان ناو هەموو (coronal, middle, apical) لە ڕووی ئامارەوە گەیشتە ئاستی گرینگ پە ێیویست .

کۆتا دەرئەنجام: بەپێی دەرئەنجامەکانی ئەم توێژینەوەیەی ئێستا, بە ڕێژەی خەستی 25 mg/mL لە هەویری دژەبەکتریای تاك دانەیی (nitrofurantoin) دەتوانێت بەتەواوەتی ئینتیرۆکۆکەس فیکالس لە ناو ببات کاتێك وەك چارەسەرکردنی لە ناو کەناڵی ڕەگی ددان بەکاردەهێنرێت , وە گونجاوە لە ڕووی بایۆلۆجیەوە لەگەڵ شانەی ژێر پێستی جرجەکان , وە هەروەها ئەبێتە هۆی کەمکردنەوەی ڕەقبونی عاجی ددان بە بەهاێیکی کەمتر بە بەراورد لەگەڵ هەویری دژەبەکتریای سیانی ئامادەکراو 25 mg/mL.

ووشە سەرەکیەکان: بەکتریای ئینتیرۆکۆکەس فیکالس, چارەسەری ناو کەناڵی ڕەگی ددان, ڕەقی ماددە, هەویری دژەبەکتریای سیانی ئامادەکراو, نایترۆفیورانتۆین (nitrofurantoin).