IN-VITRO STUDY ON ANTIBACTERIAL, CYTOTOXICITY AND pH EVALUATION OF PROPOLIS, PIPER BETLE AND CALCIUM HYDROXIDE AS INTRACANAL MEDICAMENTS

AYESHA RAFI

UNIVERSITI SAINS MALAYSIA

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AYESHA RAFI

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LIST OF SYMBOLS

˚C	Degree Celsius
×	Multiply
=	Equal
□	Percentage
µl	Microliter
®	Registered
+	plus
-	minus

LIST OF ABBREVIATIONS

Alpha- MEM	Minimum essential medium
ATCC	American Type Culture Collection
$Ca(OH)_2$	Calcium Hydroxide
CFU	Colony Forming Units
CLSI	Clinical Laboratory Standard and Institute
DMSO	Dimethyl Sulphoxide
EEPB	Ethanolic Extract of *Piper betle*
EEP	Ethanolic Extract of propolis
FBS	Fetal bovine serum
g	gram
h	hour
HPdLF	Human periodontal ligament fibroblasts
ISO	International Organization for Standardization
MBC	Minimum Bactericidal Concentration
mg/ml	Milligram per milliliter
MHA	Mueller- Hinton Agar
MHB	Mueller -Hinton Broth
MIC	Minimum Inhibitory Concentration
min	Minutes
MTT	Mosmann's Tetrazolium Toxicity assay
mg	milligram
PBS	Phosphate Buffer Saline
pH	Power of hydrogen
rpm	revolution per minute
SPSS	Statistical Package for Social Sciences
UK	United Kingdom
USA	United States of America
WEPB	aqueous extract of *Piper betle*
WEP	aqueous extract of propolis

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KAJIAN IN-*VITRO* TERHADAP PENILAIAN ANTIBAKTERIA, KESITOTOKSIKAN DAN pH KE ATAS PROPOLIS, SIREH DAN KALSIUM HIDROKSIDA SEBAGAI UBAT INTRAKANAL

ABSTRAK

Produk semula jadi seperti propolis dan sireh telah menunjukkan aktiviti antibakteria terhadap patogen oral rintang antibiotik seperti *Enterococcus faecalis (E. faecalis)* dan dianggap kurang toksik berbanding kalsium hidroksida [$Ca(OH)_2$]. Walau bagaimanapun, peranannya sebagai ubat intrakanal, aktiviti antibakteria terhadap *E. faecalis*, kesitotoksikan dan sifat berasid atau bes belum diterokai. Kajian ini bertujuan untuk menilai dan membandingkan aktiviti antibakteria pada *E. faecalis*, sifat berasid / alkali dan kesan sitotoksik propolis, sireh dan $Ca(OH)_2$ ke atas sel fibroblas periodontal manusia (HPdLF). Lima kumpulan dibentuk iaitu ekstrak etanol propolis (EEP); ekstrak etanol sireh (EEPB); ekstrak akueus propolis (WEP); ekstrak akueus sireh (WEPB) dan $Ca(OH)_2$. Selepas pembinaan semula pertumbuhan strain *E. faecalis* (ATCC 29212), ujian pencairan kaldu dilakukan untuk menentukan kepekatan perencatan minimum (MIC) dan kepekatan minimum bakterisidal (MBC). Kebolehhidupan sel pada HPdLF dilakukan pada kepekatan antara 100 mg/ml hingga 0.78 mg/ml bahan yang diuji dengan menggunakan ujian MTT. Data dianalisis dengan ujian korelasi Kruskal-Wallis dan Spearman pada taraf keertian yang ditetapkan pada 0.05 dan 0.01. MIC dan MBC terendah dan terbaik dilaporkan untuk EEP dan EEPB pada 3.12 mg/ml dan 6.25 mg/ml diikuti oleh WEPB dan $Ca(OH)_2$ dengan MIC pada 50 mg/ml dan MBC pada 100 mg/ml. MIC dan MBC tertinggi dilaporkan untuk WEP pada 200 mg/ml dan 400 mg/ml.

Purata pH untuk propolis dan sireh didapati berasid manakala $Ca(OH)_2$ adalah beralkali. Ujian MTT daripada lima kumpulan ujian pada HPdLF setelah 24 jam pada kepekatan antara 100 – 0.78 mg / ml menunjukkan bahawa EEPB, EEP, WEP dan WEPB tidak toksik kepada HPdLF berbanding dengan kawalan negatif. Kepekatan penghambatan lima puluh peratus (IC_{50}) untuk EEPB, EEP, WEP dan WEPB dianggarkan melebihi 100 mg/ml. Walau bagaimanapun, $Ca(OH)_2$ toksik pada kepekatan 100 mg/ml dan 50 mg/ml dan IC_{50} didapati pada 43.53 mg/ml. Hubungan korelasi antara pH dan kepekatan untuk propolis tidak ditemukan. Walau bagaimanapun, korelasi songsang dilaporkan untuk sireh dan korelasi langsung dilaporkan untuk $Ca(OH)_2$ ($p<0.01$). pH tidak berkaitan dengan peratusan kebolehhidupan sel untuk semua kumpulan kecuali $Ca(OH)_2$ yang melaporkan korelasi songsang ($p<0.01$). Ekstrak sireh dan propolis mempunyai aktiviti antibakteria yang berkesan terhadap *E. faecalis*, bersifat berasid dan kurang sitotoksik kepada HPdLF berbanding dengan $Ca(OH)_2$.

IN-VITRO STUDY ON ANTIBACTERIAL, CYTOTOXICITY AND pH EVALUATION OF PROPOLIS , PIPER BETLE AND CALCIUM HYDROXIDE AS INTRACANAL MEDICAMENTS

ABSTRACT

Natural products such as propolis and *Piper betle* have shown antibacterial activity against resistant oral pathogens such as *Enterococcus faecalis (E. faecalis)* and are considered to be less toxic compared to calcium hydroxide [$Ca(OH)_2$]. However, their role as an intracanal medicament, antibacterial activity against *E. faecalis*, cytotoxicity, and acidic or basic nature has not been explored. This study was aimed to evaluate and compare the antibacterial activity of *E. faecalis*, acidic/alkaline nature, and cytotoxic effect of propolis, *Piper betle*, and $Ca(OH)_2$ on human periodontal fibroblasts (HPdLF). Five test materials were used: ethanolic extract of propolis (EEP); ethanolic extract of *Piper betle* (EEPB); aqueous extract of propolis (WEP); aqueous extract of *Piper betle* (WEPB) and $Ca(OH)_2$. After the growth of the *E. faecalis* strain (ATCC 29212), broth dilution testing was performed to define the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Cytotoxicity was determined by MTT assay on HPdLF with the concentration range of 100mg/ml to 0.78mg/ml for all the test materials. The data were analysed by Kruskal-Wallis and Spearman's correlation test at the level of significance set at 0.05 and 0.01. The lowest and best MIC and MBC were reported at similar concentration for EEP and EEPB at 3.12 mg/ml and 6.25 mg/ml followed by WEPB and $Ca(OH)_2$ with MIC at 50 mg/ml and MBC at 100 mg/ml. The highest MIC and MBC were reported for WEP at 200 mg/ml and 400 mg/ml. The mean pH for propolis and *Piper betle* were found to be acidic, whilst $Ca(OH)_2$ was alkaline. MTT assay revealed that EEPB, EEP, WEP, and

WEPB were not toxic to HPdLF compared to the negative control. The fifty percent inhibitory concentration (IC_{50}) for EEPB, EEP, WEP, and WEPB was estimated above 100 mg/ml. However, $Ca(OH)_2$ was toxic at the concentration of 100 mg/ml and 50 mg/ml, and IC_{50} was found at 43.53 mg/ml. No correlation between pH and concentration for propolis was found. However, an inverse correlation was reported for *Piper betle* and a direct correlation was reported for $Ca(OH)_2$ ($p<0.01$). The pH was not related to the percentage cell viability of fibroblasts for all the groups except in $Ca(OH)_2$ which reported inverse correlation ($p<0.01$). The propolis and *Piper betle* extracts had effective antibacterial activity against *E. faecalis*, acidic, and less cytotoxic to HPdLF as compared to $Ca(OH)_2$.

CHAPTER 1
INTRODUCTION

1.1 Background of the Study

The primary cause of root canal infection is mainly due to the presence of microorganisms that may enter the canal due to various factors such as caries, trauma exposure, and tooth fracture. The procedure for a root canal treatment generally includes cleaning and debridement of the root canals, followed by the application of antimicrobial agents to get rid of the infection. Also, complex root anatomy is a challenge for the clinician as even after proper instrumentation, the bacteria tend to survive inside the canal. Systemic antibiotics are not preferred as the infected root canal is inapproachable to the local defense system due to necrosis of the root canal, and the amount of the drug reaching the canal after systemic administration of antimicrobial agents is very low to cause inhibition of bacterial species (Mohammadi & Abbott, 2009). Recently, pathogenic and non-pathogenic bacterial species in the oral cavity are showing antibiotic resistance to conventional systemic antibiotics. Additionally, adverse side effects in the form of hypersensitivity reactions are also reported after systemic administration of antibiotics (Lasemi *et al*., 2015). Therefore, administration of local drugs or intracanal medicaments in the root canal may be more appropriate for drug delivery (Kumar A *et al*., 2019; Mohammadi & Abbott, 2009).

Intracanal medicaments are chemical antiseptic agents applied to the walls of root canals during inter-appointment or after the instrumentation of the root canals. An ideal medicament should possess high biocompatibility. It should result in healing of the pulp tissues and alleviate the inflammation of the tissues instead of aggravating it (Keiser *et al*., 2000). There are various types of intracanal medicament to treat infected canals such as phenols (cresol, camphorated parachlorophenol), aldehydes

(formocresol, glutaraldehyde), halides, steroids, antibiotics, and calcium hydroxide [$Ca(OH)_2$]. The most common and preferred intracanal medicament is $Ca(OH)_2$. $Ca(OH)_2$ is a strong base with a high pH of 12.5 to 12.8 and has a wide range of antimicrobial activity. Most of the oral pathogens are unable to survive at this high pH and ultimately get eliminated from the root canal (Mohammadi & Dummer, 2011), leading to effective root canal treatment. But now there is the emergence of a few bacterial pathogens that can survive the high pH of $Ca(OH)_2$.

Enterococcus faecalis (*E. faecalis*) is a gram positive, facultative anaerobe that is present predominantly in the infected root canal. It contributes to the majority of root canal treatment failure ranging from 24 to 77% (Zancan *et al*., 2018; Tong *et al*., 2017; Frough-Reyhani *et al*., 2016; Zhang *et al*., 2015; Tennert *et al*., 2014; Murad *et al*., 2014). This pathogen was reported to be associated with most primary root canal infections (Tennert *et al*., 2014) and also prevalent in many cases of persistent endodontic infections (Łysakowska *et al*., 2016). The resistance of *E. faecalis* is due to its ability to maintain optimum potential of hydrogen (pH) level by proton pump mechanism to counteract the high pH of $Ca(OH)_2$ (Saha *et al*., 2015). The resistant properties of this bacteria are causing ineffectiveness of most of the intracanal medicament including $Ca(OH)_2$ (Abbaszadegan *et al*., 2016).

Natural products such as propolis, *Piper betle*, ginger extract, psidium, castor oil, and many more have shown antibacterial activities against pathogens of the root canal (Almadi & Almohaimede, 2018; Tabrizizadeh & Cordell, 2018; Ahangari *et al*., 2017; Valera *et al*., 2013). Currently, attempts are made to utilize the natural products in different fields of dentistry to discover the effects on various oral diseases such as oral cancer, as the intracanal medicament, periodontal tissue repair system, bonding agents, etc. (Kishan *et al*., 2020; Tabrizizadeh & Cordell, 2018; Venkateshbabu *et al*.,

2016; Tewari *et al*., 2016; Meiyanto *et al*., 2012; Carlos Groppo *et al*., 2008). Many published research on propolis, a by-product of honey tested as an intracanal medicament, pulp capping agents, storage media, and mouth rinse (C. De Carvalho *et al*., 2019; Abbasi *et al*., 2018; Sardana *et al*., 2013; Casaroto *et al*., 2010). Similarly, *Piper betle* also called perennial vine or climber was reported to possess *in-vitro* antibacterial properties (Phumat *et al*., 2018; Phumat *et al*., 2017; Khamdang *et al*., 2010). However, there are still some natural products which are not studied till now for their activities and effects when used as intracanal medicaments such as *Piper betle.*

1.2 Problem Statement

The role of intracanal medicament has become vital as they are not capable enough to result in a pathogen-free root canal system (Athanassiadis *et al*., 2010). With the decrease in efficiency of $Ca(OH)_2$ as an antibacterial agent, it has been reported to cause weakening of the tooth and cytotoxic to the fibroblasts (Cintra *et al*., 2017; Abbaszadegan *et al.*, 2016; Paramitta *et al*., 2015). $Ca(OH)_2$ has a high pH (12.5) which is responsible for the fatality of resistant bacterial species (Weckwerth *et al.*, 2013; Evans *et al.*, 2001). However, a report suggested high pH is also considered to be toxic towards the periodontal tissues (Gheorghiu *et al*., 2014). Similarly, other intracanal medicaments e.g. aldehyde-based intracanal medicaments (formocresols and iodine potassium iodide) and phenolic medicaments (eugenol and camphorated monochlorophenol) are reported to be cytotoxic to the cells in the long term of usage and are not preferred by the dentists anymore (El Karim *et al*., 2007; Chang *et al*., 2000).

1.3 Rationale of the Study

Natural products are better endured over some manufactured synthetic medicaments. Natural antimicrobial products have shown significant importance in

various antibacterial activity against microorganisms (Dzoyem *et al.,* 2018; Moloney 2016; Dias *et al*., 2012). Previously, many studies reported the antibacterial activity of propolis against *E. faecalis* (Awawdeh *et al*., 2018; Vasudeva *et al*., 2017; Kousedghi, 2012). Although, these studies differed in the test performed (agar dilution or broth dilution) and dentine or non-dentine models used. Propolis was also found to be less toxic as compared to $Ca(OH)_2$ (Jahromi *et al*., 2014; Mori *et al*., 2014), and the pH of propolis was around neutral to basic range (Fung *et al*., 2015). Surprisingly, there is only one comparative evaluation on the effect of ethanolic and aqueous extract of Iranian propolis on *E. faecalis* (Ehsani *et al*., 2013). However, no such study has been performed on Malaysian propolis where the ethanolic and aqueous extract has been compared in terms of antibacterial activity against *E. faecalis* and toxicity towards periodontal fibroblasts. The type of solvent for the extraction of natural products can influence the effectiveness of the extract. Ethanol is considered a better solvent compared to water for extraction of natural products as it can lead to a higher percentage of flavanoid and phenolic content, although, it is not preferred in paediatric and ophthalmic patients (Kubiliene *et al*., 2015).

Piper betle is abundantly found in the Asian and southeast Asian countries including Malaysia (Chakraborty and Shah, 2011). Since the olden times, *Piper betle* has been used to treat various oral diseases and has many beneficial properties such as antimicrobial, anti-carcinogenic, anti-inflammatory, and antioxidant (Karak *et al*., 2018; Ali *et al*., 2018; Haslan *et al.,* 2015). However, limited literature is reported for the antibacterial activity of *Piper betle* against *E. faecalis* (Jamelarin *et al*., 2019; Amalia and Rizki I, 2019; Khamdang *et al*., 2010) and there are currently no reports on the pH or toxicity of *Piper betle* against periodontal fibroblasts.

There is no study related to propolis and *Piper betle* in terms of intracanal medicament and comparing the performance of ethanolic and aqueous extract evaluating the antibacterial activity against *E. faecalis*, pH, and cytotoxicity on periodontal fibroblasts. These factors formed the basis for the selection of propolis and *Piper betle* as the tested natural products in this study.

1.4 Objectives

1.4.1 General objective

To evaluate the antibacterial efficacy against *E. faecalis*, pH, and cytotoxic effect of calcium hydroxide, propolis, and *Piper betle* as intracanal medicaments.

1.4.2 Specific objectives

1. To determine and compare the ethanolic extract and aqueous extract of propolis and *Piper betle* in terms of minimum inhibitory concentration and minimum bactericidal concentration with calcium hydroxide against *E. faecalis*.
2. To determine and compare the pH of the calcium hydroxide, ethanolic extract and aqueous extract of propolis and *Piper betle* at different concentrations.
3. To compare the cytotoxicity effect of calcium hydroxide, ethanolic extract and aqueous extracts of propolis and *Piper betle* of periodontal fibroblasts cells at different concentrations.

4. To investigate the co-relation between pH, concentration of calcium hydroxide, ethanolic extract and aqueous extracts of propolis and *Piper betle*, and viability of periodontal fibroblasts cells.

1.5 Research Questions

1. What is the MIC and MBC of calcium hydroxide, ethanolic and aqueous extracts of propolis and *Piper betle* against *E. faecalis*?
2. Is the pH of ethanolic and aqueous extracts of propolis and *Piper betle* acidic or basic as compared to calcium hydroxide?
3. What is the effect of calcium hydroxide, ethanolic and aqueous extracts of propolis and *Piper betle* on the viability of periodontal fibroblasts cells?
4. Is there any correlation between pH, the concentration of tested materials, and viability of periodontal fibroblasts cells?

1.6 Research Hypotheses

1. There is no antibacterial activity of propolis and *Piper betle* against *E. faecalis* compared to calcium hydroxide.
2. The pH of propolis and *Piper betle* is neutral as compared to calcium hydroxide.
3. Propolis and *Piper betle* are less cytotoxic to periodontal fibroblast cells as compared to calcium hydroxide.
4. There is no correlation between the pH, concentration of calcium hydroxide, ethanolic and aqueous extract of propolis and *Piper betle*, and viability of periodontal fibroblasts cells.

CHAPTER 2
LITERATURE REVIEW

2.1 Endodontic Infection

The root canal system consists of dental pulp which is made up of nerves and vascular tissues and safeguarded by durable dental structures like dentine, enamel, and cementum. Although the microorganisms live in harmony within the healthy oral ecosystem, when there is a pathological condition, some microorganisms dominate and result in the infection or decay of the tooth. These microorganisms can pass through the hard tissue and enter the root canal of the tooth and result in endodontic infection. The entry of microorganisms inside the root canal system can lead to initial pulpitis which may be reversible or can cause severe damage resulting in the necrosis of pulp tissues. Once there is necrosis in the pulp, the defense system is impressively compromised as the blood circulation is affected. Inflammation of this infected or necrotic pulp tissue finally results in apical periodontitis (Ørstavik, 2020).

Continuous irritation of the inflamed and necrotic pulp results in the formation of an abscess, granuloma, and cyst, and very rare cases can also be fatal. The goal of root canal treatment is to limit further infection and reduce the number of microorganisms causing the infection. However, with limited access to instruments and complex anatomy of the root canal, the main aim of endodontists is to reduce the pathogenic micro-organism to the degree that there is no further disease (Siqueira & Rocas, 2008). Invasion and colonization of the necrotic pulp by microorganisms specifically anaerobic bacterial species is the cause of primary infection (Tzanetakis *et al*., 2015; Anderson *et al*., 2013; Siqueira & Rocas, 2009).

Pirani *et al.,* (2015) conducted a retrospective study on long term outcome of root canal treated teeth and estimated a success rate of 84.7% after ten years of follow

up, whereas Hannahan & Eleazer (2008) reported outcome of root canal treatment with a success rate of 99.3 % after a follow up of twenty-two months. In disagreement with these reports, a study by Petersson *et al*., (2016) on root canal-filled teeth involving the Swedish population estimated a survival rate of only 65% after 20 years of follow-up. This report provides evidence that root canal treatment is not always successful. Failure of root canal treatment may be due to secondary infection that arises due to persistent microorganisms present in the primary infection which somehow resisted and survived the root canal treatment and period of nutrient deficiency in the treated canal (Siqueira & Rocas, 2009).

Failed cases are mainly attributed to improper instrumentation or technical faults on the part of the dentists. The causes of persistent apical periodontitis or endodontic failure are mainly related to the intra-radicular and extra-radicular pathology caused by microorganisms and other factors such as cyst, foreign body reactions, etc. (Carlos Estrela *et al*., 2014; Nair, 2004). Song *et al*., (2011) in their study found that in the cases of endodontic failure, the cause of microorganisms penetration in the treated root canal may be attributed to leakage (30.4 %), the missing canal (19.7 %), under filling, anatomical complexity, overfilling, iatrogenic problems, calculus, and cracks. The most common microorganism associated with the failed cases is *E. faecalis* (Murad *et al*., 2014; Siqueira *et al*., 2011).

2.1.1 Enterococcus faecalis

2.1.2 *E. faecalis* and its morphological and metabolic characteristics

E. faecalis is a gram-positive, non-spore forming, fermentative, facultatively anaerobic bacteria occurring in the form of cocci either in short chains, pairs, or single, non-motile (Zoletti *et al.,* 2011) and are associated with disease of various tissues includes endocardium, urinary, bloodstream, abdomen, and burns, and are considered as nosocomial (Guzman Prieto *et al*., 2016; Arias & Murray, 2012). *E. faecalis* is present in the human intestine, however, it is also responsible for the pathological condition of oral cavity especially in immunocompromised patients (Papagheorghe, 2012), failed root canal treatment (Murad *et al*., 2014; Siqueira & Rocas, 2004) and apical periodontitis (Wang *et al*., 2012).

The typical cell structure of this gram positive bacteria includes a cell wall, nuclear body or nucleoid, cytoplasmic organelles that lack the membrane and surface structures such as capsule, flagella, and pili. The cell wall of *E. faecalis* is mainly composed of peptidoglycan, teichoic acid, and lipoteichoic acid. About 90% of the cell wall is made of peptidoglycan. Peptidoglycan is a porous structure and almost all substances can traverse through peptidoglycan. It consists of repeating units of disaccharides (N-acetylglucosamine), stem (L-Ala-D-iso-Gln-Llys-D-Ala-D-Ala), and bridge (L-Ala-L-Ala) (Yang *et al*., 2017). Teichoic acid is a glycopolymer embedded in the peptidoglycan layers. Teichoic acid maintains the cell shape by providing rigidity to the cell wall. Teichoic acid is believed to provide resistance to adverse conditions such as high salt concentration, beta-lactam antibiotics, and high temperature (Brown *et al*., 2013). Teichoic acid attached to peptidoglycan is called wall teichoic acid and

teichoic acid attached to lipid is called lipoteichoic acid. Lipoteichoic acids are cytotoxic, antigenic, and adhesive temperature (Brown *et al*., 2013; Yang *et al*., 2017).

Enterococci can utilise energy sources such as carbohydrates, lactate, glycerol, citrate, malate, arginate, arginine, and keto acids (Stuart *et al*., 2006). It can persist in harsh environments such as high salt concentration and high pH. They can grow at a temperature range of 10 to 45˚C and can persist at a temperature of 60˚C for 30 minutes (John *et al*., 2015; Tendolkar *et al*., 2003). Currently, twenty-three *Enterococcus* species exist in literature which is divided into 5 groups. *E. faecalis* belongs to a group that can form acid in mannitol, arginine, and sorbose broth and it can tolerate tellurite, utilise pyruvate, and is arabinose negative (John *et al*., 2015;Stuart *et al*., 2006).

2.1.2(a) *E. faecalis* isolation and identification

E. faecalis is grown in Brain Heart Infusion and Tryptic soy agar with 5 % sheep blood at 35˚C. The colonies of *E. faecalis* obtained are subjected to several tests for identification which involve utilization of metabolites such as arabinose, tellurite, and pyruvate. Conventional techniques include gram staining, catalase test, colony morphology, hydrolysis of esculin in the presence of bile salts, growth in sodium chloride broth, hydrolysis of arginine, motility, pyruvate utilisation, carbohydrate fermentation, and pigment production tests (Zoletti *et al*., 2011).

Recently, many molecular techniques are developed for identification such as whole-cell protein, DNA-DNA hybridization, sequencing of the16S rRNA genes, gas-liquid chromatography of fatty acids. These methods mostly involve PCR amplification assays along with electrophoretic analysis of probing and sequencing PCR products or both (Zoletti *et al.*, 2011; Stuart *et al*., 2006). Pulse field gel electrophoresis (PEGE)

and Random amplified polymorphic DNA (RAPD) are utilised to evaluate variations in DNA sequences and to determine *E. faecalis* subtypes.

2.1.2(b) Involvement of *E. faecalis* in endodontic infection

There is much debate on the association of *E. faecalis* with endodontic infection. Some researchers suggested that *E. faecalis* is not a common pathogen in endodontic infections, whilst several other reports suggested the opposite of this notion (Gomes *et al*., 2015; Murad *et al*., 2014; Siqueira *et al*., 2009). This difference could be due to different sampling methods and analyses used in their studies.

The widely used techniques for the detection of *E. faecalis* in endodontic infections are culture and PCR techniques. *E. faecalis* was detected in 18.5 to 70% in failed root canal treatment and 4 to 12.5% in primary cases of endodontic infection using culture method, and 67 to 89.6% in failed treatment and 33 to 89.3% in primary endodontic infection using PCR method (Lins *et al*., 2013). In another study using pyrosequencing technology reported that *E. faecalis* was found in lower percentage (0.7%) as compared to the other bacterial species in primary and persistent endodontic infection. However, this study did not consider the samples from the biofilm and coronal leakage cases (Hong *et al*., 2013). A similar finding was reported by Keskin *et al.*, (2017) that *Enterococcus* was less abundant compared to another genus using pyrosequencing technology. The limitation of this study was that it did not consider the cases of severe periodontal disease which may be the cause of a low percentage of *E. faecalis*.

Contradicting these two reports, Gomes *et al.*, (2015) investigated the microbiomes of the endodontic-periodontal lesion using Next Generation Sequencing and reported that *E. faecalis* was one of the most frequently detected species along with

Parvimonas micra, Filifactor alocis, Mogibacterium timidum, and *Fretibacterium fastidiosum* before and after chemomechanical preparation. Several other studies reported the presence of *E. faecalis* in the association of either the primary endodontic infections (4-40%) or secondary/persistent endodontic infections (24-77%) (Ferreira *et al.,* 2015; Murad *et al.,* 2014; Tennert *et al*., 2014; Ozbek *et al*., 2009; Stuart *et al*., 2006; Rocas *et al*., 2004).

2.1.2(c) *E. faecalis* and its association with failed root canal treatment

E. faecalis is one of the most common pathogens isolated from failed root canal treatment cases (Pourhajibagher *et al*., 2017; Murad *et al.,* 2014; Ozbek *et al.,* 2009). Ozbek *et al.,* (2009) found that *E. faecalis* was present in 74.4% of root-filled teeth/secondary infection as compared to 25% of primary endodontic infections in the Turkish population using real-time PCR technique, indicating that *E. faecalis* is mainly associated with the failed cases/secondary endodontic infections. *E. faecalis* was also more dominant in secondary endodontic infection cases (36.6%) as compared to primary endodontic infections using biochemical tests and RNA gene sequencing method (Pourhajibagher *et al*., 2017). Murad *et al*., (2014) in their study found that *E. faecalis* was the most prevalent species (28%) in persistent endodontic infection using checkerboard DNA-DNA hybridization. Similar findings were also reported in other studies that *E. faecalis* are more commonly associated with secondary endodontic infections (Pirani *et al*., 2008; Foschi *et al*., 2005). Dumani *et al*., (2012) however, reported the presence of *E. faecalis* in 16% of necrotic pulp tissues/ primary endodontic infection as compared to 10% in retreatment cases/secondary endodontic infection, indicating no significant difference between the association of *E. faecalis* with primary and secondary infections.Besides, *E. faecalis* was also reported resistant to the different

types of antibiotics (Barbosa-Ribeiro *et al*., 2016; Ferreira *et al*., 2015; Miller *et al*., 2014) which is discussed in next paragraph.

2.1.2(d) Resistance of *E. faecalis* to antibiotics

In-vitro and *in-vivo* studies found that *E. faecalis* was resistant to several intracanal medicaments including tetracycline, metronidazole, erythromycin, clindamycin, ciprofloxacin, minocycline, and chlorhexidine (Barbosa-Ribeiro *et al*., 2016; Ferreira *et al*., 2015), clindamycin, gentamycin, rifampicin, and vancomycin (Periera *et al.*, 2017). Barbosa-Ribeiro *et al*., (2016) in their study reported that *E. faecalis* displayed various degrees of resistance (intermediate/total) to various antimicrobial agents and almost all of the antibiotics were ineffective except amoxicillin + clavulanic acid using E-test method. *E. faecalis* was the most frequent bacterial species found after instrumentation and root canal treatment with $Ca(OH)_2$ and a mixture of $Ca(OH)_2$ and chlorhexidine in primary endodontic infection (Ferreira *et al.*, 2015). It has been suggested that survival of *E. faecalis* may be due to various reasons such as antibiotic resistance, virulence factors, resistance to high pH and biofilm formation.

E. faecalis offers resistance to antibiotics acting on the cell wall such as ampicillin, penicillin, cephalosporin by altering the sequence of the protein and amino acids (Miller et al., 2014; Rice *et al.*, 2004). *E. faecalis* also display resistance to antibiotics which primarily interfere with the protein synthesis such as aminoglycosides, linezolid, macrolides by modification of hydroxyl and amino group with the assistance of *Enterococcal* enzymes or mutation in the genes encoding nucleic acids (Miller *et al.*, 2014). Antibiotics interfering with the nucleic acid replication,

transcription, and synthesis such as quinolones, rifampicin, trimethoprim are offered resistance by altering the binding affinity of these drugs through mutation in the target genes (Lopez *et al.,* 2011; Miller *et al.,* 2014).

2.1.2(e) Resistance of *E. faecalis* due to virulence factors

Virulence factors promote adherence to host cells, assist in tissue invasion, immune modulation and cause damage through secretion of toxins (Mishra *et al*., 2017; Zou & Shankar, 2016). These factors include *Enterococcal* surface protein (ESP), toxins (hemolysin, cytolysin, gelatinase, aggregation substances), cell wall polysaccharides, pheromones, lipoteichoic acids.

ESP is believed to help the bacteria in persistence and colonisation during infection through biofilm formation and it maintains the primary contact of the pathogen with the host surface and helps in the adherence of bacterial cell to the host through uroplakin or mucin (Zou & Shankar, 2016; Zoletti *et al*., 2011). Subsequently, toxins such as hemolysin are responsible for the lysis of human erythrocytes and promote the spread of infection (Mishra *et al*., 2017). Similarly, cytolysin causes the lysis of the cells (Van Tyne *et al.,* 2013). Gelatinase, on the other hand, promotes the degradation of fibrinogen and collagen. It can also produce collagen-binding protein like serine protease (Mishra *et al.,* 2017). The increase of *E. faecalis* adhesion to dentine *in-vitro* was associated with the gelatinase gene (Guneser & Eldeniz, 2016). Gelatinase gene also promotes biofilm formation (Tsikrikonis *et al.,* 2012).

Aggregation substances induce pheromone to promote bacterial conjugation. It helps donor enterococcal contact to the recipient to cause plasmid transfer in *E. faecalis*. Pheromones are hydrophobic peptides that function by conducting signals between *E. faecalis* cells. Antimicrobial resistance and virulence can be signalled among *E. faecalis*

strains through the pheromone system (Hirt *et al*., 2018). Aggregation substance helps the *E. faecalis* to adhere to the host by binding to the host collagen and promotes the formation of biofilm which is resistant to antibiotics (Kafil & Mobarez, 2015). Furthermore, aggregation substances protect the cell from phagocytosis and increase the hydrophobicity of the cell surface. It was reported to promote the intracellular survival of phagocytosed *E. faecalis* present in the human macrophages (Halkai *et al*., 2012). All these virulence factors help in the survival and colonization of *E. faecalis* in the root canal.

2.1.2(f) Resistance of *E. faecalis* due to pH factors

Another factor for *E. faecalis* survival is its ability to persist in altered pH conditions (van der Waal *et al*., 2011; Evans *et al*., 2001). Research on the mechanism of *E. faecalis* persistence in high pH of calcium and sodium hydroxide revealed that *E. faecalis* was able to survive at pH ranging from 9.5 to 11.5 (Weckwerth *et al*., 2013; Evans *et al.,* 2001). The cause of resistance to pH is believed to be the proton pump of the bacterial cell which drives the positive potassium ions inside the cell to cause an acidic environment when negative hydroxyl ions enter the cytoplasm of the bacteria (Evans *et al.,* 2001). An alternate mechanism is that in the case of pH higher than 8 there is an increase in Na+ K+ -ATPase activity as well as a change in cell surface hydrophobicity to resist high pH (Ran *et al.,* 2013).

2.1.2(g) Resistance of *E. faecalis* due to biofilm formation

Another important factor for *E. faecalis* survival is biofilm formation (Estrela *et al*., 2009). Biofilm is a layer of slime made of protein, polysaccharides, and microbes giving rise to the formation of a matrix that gives protection to bacterial species from

antimicrobial agents or host defence mechanism (Flemming *et al.,* 2016; Stewart & Costerton, 2001). Biofilm is surrounded by planktonic bacterial species which either leave it or adhere to biofilm. Biofilm bacteria are 1000 times more resistant to phagocytosis, antibacterial agents, and antibodies (Neelakantan *et al.*, 2017; Devaraj *et al.,* 2016) as compared to planktonic cells. Resistance due to biofilm can be attributed to the structures present on the cell surface (e.g. capsule) or secretions (e.g. extracellular polysaccharides). ESP can protect the bacteria from the environment such as high pH, UV radiation, osmotic shock, and desiccation. It also reduces the concentration of substances that pass-through the EPS matrix before reading the bacteria (Neelakantan *et al.,* 2017).

Biofilms provide several benefits to microorganisms especially antimicrobial resistance and allow the microorganisms to multiply on their surface by protecting host defense and toxic substances as the carbohydrate/polysaccharide matrix of the biofilm act as a physical barrier against the external environment (Flemming, 2016; Jett *et al*., 1994). The other benefit is that the physiology of microorganisms present in the biofilm is modified and microorganisms living in the biofilm multiply slowly in comparison to planktonic cells, which finally result in the slow uptake of chemical antibacterial substances (Neelakantan *et al.,* 2017; Elsner *et al.,* 2000). The heterogeneous environment i.e., cells which are present deep in the biofilm face different environmental condition than those present at the surface. This heterogeneous composition causes altered phenotypes (Ten Cate, 2012). Some researchers found that the presence of a sub-population of microorganisms within the biofilm causes resistance to antimicrobial agents (Zhao *et al.,* 2016; Kaldalu *et al*., 2016). Biofilms also help in the uptake of nutrients (Simain *et al.,* 2010), thereby assisting the bacterial species to survive in harsh environments.

In conclusion, the above factors are responsible for the resistance of *E. faecalis* and therefore is a cause of concern for the endodontists.

2.2 Intracanal Medicament

Many modalities have been suggested to solve the above-mentioned problems and one of them is intracanal medicaments. Intracanal medicaments are the chemical substances or antimicrobial agents placed temporarily after biomechanical preparation in root canal treatment (Lima *et al.,* 2012). However, there is much ongoing debate on the role of intracanal medicament and its necessity.

A study on the antibacterial efficacy of different intracanal medicaments such as $Ca(OH)_2$, chlorhexidine (CHX), and the mixture of $Ca(OH)_2$ and CHX found that bacterial load was decreased after instrumentation, however, there was no significantly difference between the samples before and after application of intracanal medicaments for one week (Manzur *et al.,* 2007). Endo *et al*., (2013) investigated bacterial pathogens present in root-filled teeth and post-treatment apical periodontitis by colony-forming units. Fifteen root-filled teeth were studied with their gutta-percha removed and divided into three groups. The medications used were $Ca(OH)_2$+CHX, $Ca(OH)_2$+sodium chloride, and CHX gel. The results were recorded for samples with medication (for one week and 14 days) and without intracanal medicament. It was found that there was no statistically significant difference between the sample with and without medicament which indicated that intracanal medicament did not cause disinfection of the root canal. In agreement with these two types of researchers, and *in-vivo* study on antibacterial effectiveness of CHX, $Ca(OH)_2$, and metronidazole against aerobic and facultative anaerobic microorganisms, found that all of these three medicaments were ineffective in eliminating the microorganisms from human primary teeth having necrotic pulp

(Paikkatt *et al.*, 2018). However, the limitation of all of these studies was a small sample size and duration of sampling or time frame, which should include different periods.

Contradicting these studies, Silveira *et al.* (2011) suggested that $Ca(OH)_2$, 2% CHX, $Ca(OH)_2$ + chloromonochloramphenicol (CMCP) + propylene glycol, $Ca(OH)_2$ and propylene glycol, $Ca(OH)_2$ + saline exhibited antibacterial activity against *Staphylococcus aureus, E. faecalis, Streptococcus mutans (S. mutans) and Pseudomonas aeruginosa* using broth dilution method. Another study found that CHX gluconate gel was the most effective against *E. faecalis, S. mutans, and C. albicans* in the root canal, followed by $Ca(OH)_2$ and antibiotic corticosteroid paste (Attia *et al.*, 2015). Chua *et al.*, (2014) reported that triple antibiotic paste (TAP) i.e. 2% chlorhexidine gel, $Ca(OH)_2$ with propylene glycol and propolis were effective against *C. albicans.*

Many more studies have supported the fact that intracanal medicaments are required in between the appointments (Valverde *et al*., 2017; de Lucena *et al.*, 2013; McGurkin-Smith *et al*., (2005) and can effectively reduce the bacterial load to an extent that can be tolerated by pulp and periapical tissues, leading to successful root canal treatment. If the canal is not treated after instrumentation and in between the appointment, the bacterial population might multiply and grow to reach the original level as it was before the instrumentation (Chong & Ford, 1992). Root canal medicament prevents the leakage from the canal and creates an inert atmosphere inside the canal by eliminating the microorganisms, neutralizing the debris from dead tissues, and drying the wet canals. Therefore, two ways are suggested by which intracanal medicament prevent the entry of bacterial species from saliva. First, the intracanal medicament work as a barrier, chemically by destroying bacteria to prevent their penetration into the root canal (Pavaskar *et al.*, 2012; Silveira *et al*., 2011). Secondly,

medicaments act as a physical barrier against the entry of bacterial species by filling the complete length of the root canal. Other than acting as an antibacterial agent, intracanal medicaments are believed to reduce the infection, pain, and inflammation of the pulp (Prasad *et al.,* 2016; Eftekhar *et al.,* 2013).

Eftekhar *et al.,* (2013) conducted a randomised clinical trial on 120 patients to study the analgesic effect of corticosteroid containing compound and odontopaste (zinc oxide based root canal paste) in between the appointment for root canal therapy. It was found that pain on percussion in the group who received odontopaste and corticosteroid compound medicaments were lesser compared to placebo after 24 hours. However, there was no significant difference after 7 days. In another study that involved 30 patients, it was reported, $Ca(OH)_2$ and TAP effectively reduced inter-appointment pain even after 7 days with TAP being better than $Ca(OH)_2$ (Prasad *et al.,* 2016).

The above-mentioned studies supported the fact that intracanal medicament plays an important role in endodontic treatment. $Ca(OH)_2$ is the most commonly used intracanal medicament in clinical practice. In 1920 Hermann introduced $Ca(OH)_2$ as a direct pulp capping agent. $Ca(OH)_2$ is an odourless white powder with a molecular weight of 74.08. It acts as an insulator and is biocompatible to the pulp tissues with a compressive strength of 138. It has low solubility which decreases as the temperature increases. The dissociation co-efficient of $Ca(OH)_2$ controls the calcium and hydroxyl ion release (Mohammadi & Shalavi, 2012; Spångberg *et al*., 1979). It is a strong base and has a high pH ranging from 12.5 to 12.8 (Mohammadi & Dummer, 2011). $Ca(OH)_2$ is bacteriostatic and mildly irritating to the pulp tissues which makes it a preferred material for restoration. It is insoluble in alcohol. Aqueous medium or water is the most preferred vehicle for $Ca(OH)_2$ due to its dissociation property.

The importance of $Ca(OH)_2$ in endodontics is because of its antibacterial activity, its effectiveness in the foundation of calcified tissue, and its ability to cause protein denaturation helping in the dissolution of pulp remnants. Currently, $Ca(OH)_2$ is the common and effective intracanal dressing in endodontics (Mohammadi & Dummer, 2011).

2.2.1 Antibacterial activity of calcium hydroxide

The antimicrobial activity of $Ca(OH)_2$ is due to its dissociation into hydroxyl and calcium ions when in contact with water (Mohammadi *et al*., 2012). Hydroxyl ions are oxidant free radicals having high reactivity with the biomolecules (Lipinski, 2011) and rarely diffuses from the origin of generation. A high concentration of hydroxyl ions causes chemical destruction to the organic components (phospholipids and protein) and disturbs the transport of nutrients, ultimately altering the pH gradient and integrity of the cytoplasmic membrane (Baranwal *et al.,* 2016; Estrela *et al*., 1999). Many cell functions and cellular enzymes necessary for cell function and metabolism can be affected by the pH (Putnam, 2012). These enzymes present outside and inside of the cell wall are targeted by the hydroxyl ions released by the $Ca(OH)_2$ in an aqueous environment, thereby resulting in antibacterial activity (Estrela *et al*., 1995).

The effectiveness of $Ca(OH)_2$ as an intracanal medicament is directly related to the diffusion of hydroxyl ions through the dentine. Nerwich and Figdor (1993) reported that there was a difference in the rate of diffusion of hydroxyl ions with apical dentine having low pH compared to the cervical dentine. It was due to the increased diameter and density of dentinal tubules in the cervical part as compared to the apical part of the root. In the same study, it was reported that 7 days were required for the hydroxyl ions to diffuse the outer dentine and the peak level of hydroxyl ion diffusion took place in 3 to 4 weeks. $Ca(OH)_2$ with distilled water and RC Cal (Prime dental product, Mumbai,

India) effectively raised the pH to 12.7 and 11.8 after a week of application (Fulzele *et al*., 2011). A similar study reported that the highest hydroxyl ion release by $Ca(OH)_2$ saline paste was on day 3 and day 30, however, 7 days were insufficient for $Ca(OH)_2$ saline paste to inhibit *E. faecalis* growth (Zancan *et al.*, 2016). Another study demonstrated that pH of $Ca(OH)_2$ was higher as compared to the other medicaments i.e. chlorhexidine, propylene glycol, bioactive glass, and niobium phosphate bioactive glass after 10 minutes, 14, 21, and 30 days (Carvalho *et al*., 2016).

Dentine possesses buffering action in which H_2PO^{4-}, H_2CO^3 and HCO^{3-} proton donors present in mineral-laded hydroxyapatite reduce the antimicrobial action of $Ca(OH)_2$. It was found that dentine powder reduced the antibacterial activity of $Ca(OH)_2$, sodium hypochlorite, chlorhexidine acetate, and iodine potassium iodide at 1 and 24 hours (Haapasalo *et al*., 2000). In another study, the pH of $Ca(OH)_2$ was reduced after 14 days when dentine powder was added to root canal walls (Agrafioti *et al.*, 2013). Nevertheless, Carvalho *et al*., (2015) found that the application of dentine powder on the simulated canals did not influence the pH of 2% chlorhexidine gel, $Ca(OH)_2$, $Ca(OH)_2$+propylene glycol, and distilled water+bioactive niobium phosphate glass. There is still uncertainty on the buffering action of dentine as it adds more damage to the antibacterial activity of $Ca(OH)_2$, however, the diffusion of hydroxyl ions should exceed the dentine's buffering ability to kill the microorganisms and act as an effective antibacterial agent.

Another concern is the reduced action of $Ca(OH)_2$ against *E. faecalis* which is known to persist in high pH conditions (Weckwerth *et al.*, 2013). A susceptibility test utilising the well diffusion method to determine the antimicrobial activity of Curcuma longa, Tachyspermum ammi, $Ca(OH)_2$, and CHX gluconate gel against *E. faecalis* reported that $Ca(OH)_2$ showed a smaller zone of inhibition compared to Curcuma longa

and CHX gel (H. Kumar, 2013). Similarly, the microdilution method reported $Ca(OH)_2$ alone was less active as an antibacterial agent compared to other medicaments in a study that compared the antibacterial activity of proton pump inhibitor (PPI), TAP, $Ca(OH)_2$ against *C. albicans*, and *E. faecalis* (Mehta *et al*., 2017)

In-vitro research on extracted teeth to calculate the colony-forming units (CFUs) reported that TAP was better than $Ca(OH)_2$ after 21 days and reduced the CFU in both time and depth (Adl *et al.,* 2014). In another study, $Ca(OH)_2$ exhibited lower antibacterial activity against *E. faecalis* compared to 2% CHX, honey, propolis, and curcuma longa as intracanal medicaments (Vasudeva *et al*., 2017). However, Hemadri (2011) found that $Ca(OH)_2$ was less effective in eradicating *E. faecalis* as an intracanal medicament as compared to Nisin, an antimicrobial peptide. A similar finding was reported by Abbaszadegan *et al.,* (2016) who found that $Ca(OH)_2$ was unable to eradicate planktonic *E. faecalis* after 24 hours and biofilm *E. faecalis* after 14 days. The decrease in antibacterial effectiveness of $Ca(OH)_2$ is a setback for endodontists and along with this problem, $Ca(OH)_2$ also causes cytotoxicity to the fibroblasts cells.

2.2.2 Cytotoxicity of calcium hydroxide

A study using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay reported that *in-vitro* application of $Ca(OH)_2$ at 62.5 µg/ml resulted in Vero fibroblast cell death (Paramitta *et al*., 2011). Jahromi *et al.,* (2014) found that 1 mg/ml of $Ca(OH)_2$ resulted in 11.34% of viable fibroblast cells, as compared to 1 mg/ml propolis which resulted in 75.2% of cell viability.

Contrary to the previous studies, Yadlapati *et al*., (2014) evaluated the cytotoxic effect of TAP, double antibiotic paste, $Ca(OH)_2$, and minocycline on HPdLF by multi-parametric cytotoxic kit (XTT {2,3-bis[2-methoxy-4-nitro-5- sulfopheny]-2H-tetrazolium-5-carboxyanilide inner salt}, neutral red (NR) and crystal violet dye elution

(CVDE) assays) and found that TAP and minocycline were more cytotoxic with less than 70% viability in comparison to $Ca(OH)_2$ and DAP (Yadlapati *et al.,* 2014). However, a study by Hosseini *et al.,* (2015) on the action of TAP and $Ca(OH)_2$ on fibroblasts cells at different concentration utilising methyl tetrazolium (MTT) assay reported that 0.1 mg/ml of $Ca(OH)_2$ was non-toxic whereas 1 mg/ml and 10 mg/ml of $Ca(OH)_2$ was severely toxic to fibroblasts cells. On the other hand, TAP was mildly cytotoxic at 0.1 mg/ml and 1 mg/ml but moderately cytotoxic at 10 mg/ml to the fibroblasts cells (Hosseini *et al.,* 2015). A similar study on L929 fibroblasts cells by MTT assay reported that Otosporin and $Ca(OH)_2$ after 7 days of the application were cytotoxic to the fibroblasts cells (Farias *et al.,* 2016). Cytotoxicity of $Ca(OH)_2$ was attributed to its high alkalinity (pH 11-12) causing the necrosis of the cells as reported in a study on Calxyl® (OCO Praparate) which was highly toxic on the fibroblasts ICP-23 compared to other medicaments such as Ledermix (Reimser), Cresophene (Septodont, UK) and R4 (Septodont, UK) (Gheorghiu *et al*., 2014).

2.2.3 Dentine strength and calcium hydroxide

$Ca(OH)_2$ reduces the strength of dentine as prolonged use for 7 to 84 days reduced the micro tensile strength of the tooth by nearly 23-43.9% due to its strong alkalinity (Rosenberg *et al.,* 2007). Placement of $Ca(OH)_2$ for 30 days in root canals has been reported to decrease the compressive strength of the dentine to about 15% (Sahebi *et al*., 2010). A similar study reported that long-term application of $Ca(OH)_2$ on extracted human teeth for a period of 30, 90, 180, and 540 days showed a significant reduction in the strength of dentine after 180 days (Batur *et al.,* 2013).

2.2.4 Natural products as the alternative option

Although $Ca(OH)_2$ is the most preferred intracanal medicament, but the drawbacks suggested that it is not completely reliable in the endodontics which compels

us to explore new intracanal medicaments from natural sources as these are more biocompatible and possess better antimicrobial activity. Researchers have found many natural products such as cinnamon essential oil, Triphala, green tea, *Psidium cattleianum*, ginger extract, aloe vera, Arctium lappa to be effective antibacterial agents against resistant microorganisms of root canal including *E. faecalis* (Sangalli *et al*., 2018; Pirvu *et al*., 2017; Abbaszadegan *et al*., 2016). Natural products have shown the capability to act as an intracanal medicament and more research is required before they can be adopted in clinical practice.

2.3 Propolis

Propolis is a wax-like resinous substance that is gathered by the bees from tree buds and plants, mixed with their saliva to be used in their hives as adhesives (Simone-Finstrom & Spivak, 2010). Since time immemorial, propolis is a part of folk medicine for treating various illnesses. Propolis is as old as honey and has been used by ancient Egyptians, Romans, and Persians (Kuropatnicki *et al*., 2013).

2.3.1 Chemical composition and method of extraction

Propolis is a lipophilic substance, hard and brittle but becomes soft and sticky when the heat is applied. Colour may vary from yellow-green to reddish and dark brownish (Bankova *et al*., 2000). Generally, propolis constitutes 30% waxes, 50% resins, 10% essential oils, 5% organic compounds, and 5% pollen (Wagh, 2013). Around 300 constituents were discovered in different samples and are still being discovered. Propolis with different geographical origin has different biological activity under the influence of different climatic conditions (Woźniak *et al*., 2019; Huang *et al*., 2013; Bankova *et al*., 2000). Compounds responsible for biological activities are aromatic acids, polyphenols, and diterpenic acids. It is believed that the antibacterial