PIPER BETLE COMBINATION WITH AH PLUS AND BIOROOT RCS: PHYSICOCHEMICAL, CYTOTOXIC AND ANTIBACTERIAL PROPERTIES

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA	Analysis Of Variance
AH	AH Plus
BHI	Brain Heart Infusion
BR	BioRoot RCS
CFU	Colony Forming Units
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
EEPB	Ethanolic Extract of *Piper betle*
FBS	Fetal Bovine Serum
G	Gram
h	Hour
HPdLF	Human Periodontal Ligament Fibroblasts
ISO	International Organization for Standardization
MDCT	Modified Direct Contact Test
mg/ml	Milligram Per Milliliter
MTT	Mosmann's Tetrazolium Toxicity Assay
OD	Optical Density
PB	*Piper betle*
PBAH	Pb And AH Plus
PBBR	Pb And BioRoot RCS
PBS	Phosphate Buffer Saline
PCV	Percentage of cell viability
pH	Power Of Hydrogen
RCT	Root canal therapy
SPSS	Statistical Package for Social Sciences

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GABUNGAN SIREH (PIPER BETLE) DENGAN AH PLUS DAN BIOROOT RCS: SIFAT FIZIKOKIMIA, SITOTOKSIK DAN ANTIBAKTERIA

ABSTRAK

Penerokaan dalam bahan dan prosedur endodontik telah dibuat untuk memaksimumkan penyingkiran mikroorganisma dalam sistem saluran kanal dan seterusnya meningkatkan kadar kejayaan rawatan. Sifat fizikokimia dan antibakteria adalah matlamat dalam menghasilkan pengedap endodontik yang baru. Rintangan terhadap antibiotik boleh menyebabkan krisis kesihatan global jika kita menggunakan antibiotik secara tidak wajar. Oleh itu, penggantian kepada antibiotik diperlukan. *Piper betle* (PB) adalah salah satu herba ubatan yang mungkin boleh digunakan untuk keberkesan sifat antibakteria terhadap *E. faecalis*. Oleh itu, kajian ini bertujuan untuk menyiasat sifat fizikokimia (pH dan keterlarutan), sitotoksisiti, dan kesan antibakteria ekstrak etanol PB dalam kombinasi dengan AH Plus (AH) dan BioRoot RCS (BR) dan pengedap AH dan BR sahaja. Empat bahan kajian yang digunakan; AH Plus (AH); PB dan AH plus (PBAH); BioRoot RCS (BR); dan PB dan BioRoot RCS (PBBR). Ujian pH telah dijalankan untuk sampel segar dan set untuk tempoh dari serta-merta sehingga 4 minggu. Ujian keterlarutan adalah sejajar dengan kaedah International Standard Organization (ISO) 6876. Sitotoksisiti ditentukan oleh ujian MTT pada HPdLF pada masa yang berbeza iaitu 24, 48, dan 72 jam. Kesan antibakteria dinilai dengan ujian 'Modified Direct Contact Test' (MDCT) terhadap *E. faecalis* pada keadaan segar, set hari-1 dan set hari-7 untuk bahan yang diuji. Data dianalisis menggunakan SPSS versi 25 dengan ujian pasca Tukey pada taraf keertian $P = 0.05$. Dalam keadaan segar, PB meningkatkan kealkalian AH ($P = 0.000$) selepas 2 jam. BR dan PBBR menunjukkan nilai alkali tinggi ($P = 0.000$) pada setiap masa, tanpa perbezaan dengan kehadiran PB.

Untuk sampel set, PB menurunkan nilai pH BR manakala meningkatkan nilai pH AH. PBBR menunjukkan pengurangan dalam keterlarutan berbanding BR, manakala AH dan PBAH tidak menunjukkan perbezaan. Selain itu, PB hanya menunjukkan penurunan ketara dalam sitotoksisiti AH pada 48 jam berbanding AH (45.20%, 18.85%). PB mengurangkan kesan antibakteria BR pada tahap ketara di setiap masa (P = *0.000*, *0.014*, *0.032*). Kesimpulannya, PBAH mempunyai nilai pH yang lebih tinggi daripada AH pada keadaan segar, manakala PBBR mempunyai nilai pH yang lebih rendah daripada BR apabila set. PBBR mempunyai keterlarutan yang lebih rendah daripada BR. Berbanding dengan AH, PBAH mempunyai pengurangan sitotoksisiti yang ketara kepada HPdLF pada 48 jam. PBBR adalah sitotoksik kepada HPdLF pada 24 dan 48 jam. Kedua-dua AH dan PBAH menunjukkan sifat antibakteria, diikuti oleh BR dan seterusnya PBBR. Kesimpulannya, PBAH ialah pengedap saluran akar yang berpotensi dipertingkatkan dan boleh dikaji lebih lanjut untuk mendapatkan sepenuhnya sifat mendalamnya.

PIPER BETLE COMBINATION WITH AH PLUS AND BIOROOT RCS: PHYSICOCHEMICAL, CYTOTOXIC AND ANTIBACTERIAL PROPERTIES

ABSTRACT

Exploration in endodontic material and procedure had been made to maximise the elimination of the microorganisms in the root canal system to improve the treatment success rate. Physicochemical and antibacterial properties are important in producing a novel endodontic sealer. Antibiotic resistance can cause a global health crisis if we use them inappropriately hence alternative is needed. *Piper betle* (PB) is one possible medicinal herb to be used for its effective antibacterial properties against *Enterococcus faecalis (E. faecalis)*. Therefore, this study aims to investigate physicochemical properties (pH and solubility), cytotoxicity, and antibacterial effects of ethanolic extract of PB in combination with AH Plus (AH) and BioRoot RCS (BR) and sealers alone. Four tested materials were used; AH; PB and AH plus (PBAH); BR; and PB and BioRoot RCS (PBBR). pH testing was conducted for fresh and set sample for a period from immediate up to 4 weeks. Solubility test was in alignment with the International Standards Organization (ISO) method. At 24, 48, and 72 hours, MTT assay on HPdLF was performed to measure the cytotoxicity. Antibacterial effect was evaluated with modified direct contact test (MDCT) against *E. faecalis* at fresh, day-1-set and day-7-set state of the tested materials. Data were analysed with SPSS version 25 with Tukey's post-test at the level of significance of $P = 0.05$. In fresh state, PB increase the alkalinity of AH at significant level ($P = 0.000$) after 2 hours. BR and PBBR demonstrated significantly high ($P = 0.000$) alkaline value at all time points, without significant difference with presence of PB. For set sample, PB decrease the pH value of BR while increase the pH value of AH. PBBR showed significant

reduction in solubility as compared to BR, AH and PBAH showed no significant difference. Besides, PBAH showed significant decrease in cytotoxicity at 48 hours as compared to AH (45.20%, 18.85%). PB reduce the antibacterial effect of BR at all times at significant level (*P = 0.000, 0.014, 0.032*). Conclusion, PBAH has higher pH value than AH at fresh state, while PBBR has lower pH value than BR when set. PBBR has significant lower solubility than BR. PBAH has significant reduced cytotoxicity as compared to AH on HPdLF at 48 hours. PBBR was cytotoxic to HPdLF at 24 and 48 hours. Both AH and PBAH exhibited antibacterial property against *E. faecalis*, followed by BR and PBBR. In conclusion, PBAH is a potential enhanced root canal sealer and may be further studied to fully elicit its profound properties.

CHAPTER 1

INTRODUCTION

1.1 Background

The factors that contribute to the success of endodontic treatment are cleaning and shaping, disinfection and three-dimensional sealing of the root canal system (Tomer *et al.*, 2021). The complete sealing of the root canal system is one of the most important aspects that leads to successful endodontic treatment (Benkar Sharad, 2011). During obturation, a root canal sealer is used to create a completely sealed root canal system by closing every small gap between the gutta-percha and dentinal wall, also to seal the bacteria from the oral environment and prevent them from reaching the periapical tissue (Colombo *et al.*, 2018). Elimination of infectious microorganisms is accomplished by chemo-mechanical preparation of the root canals and intracanal medicament. However, microorganisms inside the dentinal tubule might survive the challenges and cause the infection to persist. Hence, a root canal sealer with good sealing ability and antibacterial content can be handy in this matter and ensure a better success rate of the root canal treatment (Sharma *et al.*, 2014). *Enterococcus faecalis (E. faecalis)* is highly recalcitrant in nature, hard to eradicate, and causes recurrent root canal treatment failure (Khalifa *et al.*, 2016). It has been reported that it is difficult to eliminate *E. faecalis* once it establishes itself in the dentinal tubules. Therefore, it will be beneficial to patients if a sealer with antibacterial properties is used during the obturation as the last regime in root canal treatment (Sharma *et al.*, 2014).

There are numerous different types of root canal sealers in the market, based on a comprehensive review of the current endodontic sealers (Komabayashi *et al.*, 2020), tricalcium silicate-based sealer has the most superior antibacterial properties as compared to zinc oxide eugenol sealer and epoxy resin-based sealer, while silicone-based sealer is not antibacterial. In the current days, AH Plus; a type of resin-based sealer has been the commonly most used sealer by all, due to its antibacterial effect due to the release of formaldehyde, epoxy, and amine content during the polymerization process (Nirupama *et al.*, 2014). Besides, salicylate-based sealers which are usually marketed by their therapeutic additives such as calcium hydroxide also provides antibacterial quality. Regrettably, its clinical effect is undesirable as others (Komabayashi *et al.*, 2020). There is also research on the efficacy of the combination of antibiotics with sealers where a significant increase in antibacterial properties was observed (Sharma *et al.*, 2014).

Herbal extracts are an alternative treatment regime as they contain novel bioactive compounds that have antibacterial, anti-inflammatory, sedative, and anxiolytics effects (Buggapati, 2016). *Piper betle* (PB) is a famous medicinal plant and it is a well-known plant of the *Piperaceae* family, mainly distributed in South East Asian (Azahar *et al.*, 2020). In a study done by Bhayya *et al.*, (2021) where an extract of PB as irrigating solution, PB shows an effective antibacterial and antifungal activity against *E. faecalis* and *Candida albicans (C. albicans)*. PB extract has also been reported to have antibacterial, antifungal, antioxidant, antidiabetic and anticancer properties (Umar *et al.*, 2018). Currently, PB has been incorporated in dental toothpaste (Ali *et al.*, 2018) as well as mouthwash (Gita, 2020) and it shows promising antibacterial properties in the toothpaste; demonstrates effective anti-inflammatory, anti-plaque properties when including PB extract in mouthwash, hence it is useful in supportive periodontal therapy.

An ideal root canal sealer needs to have an excellent seal when set, dimensional stability, a slow setting time to ensure sufficient working time, insolubility to tissue fluids, adequate adhesion with canal walls, and biocompatibility. These will ensure the success of root canal therapy. The ability of the sealer to enclose the bacteria is dependent on its ability to seal, which has a strong association with its solubility (Espir *et al.*, 2016). Root canal sealer's better sealing capacity may be attributed to its lesser solubility. Besides solubility that is linked with the sealer’s sealing ability, the pH value of the sealer is also another physicochemical property that plays a role in antibacterial activity. A high alkalinity sealer material will produce an antibacterial effect when the hydroxyl ions dissociate upon contact with water (Lin *et al.*, 2020). Furthermore, this high pH value will also contribute to hard tissue formation and prevent osteoclast activity (Siboni *et al.*, 2017) which may fasten the healing process of periapical tissue (Rodrigues *et al.*, 2013). The present study aims to evaluate the cytotoxicity, antibacterial activity of PB extracts combined with sealers against *E. faecalis* and some of their physicochemical properties, specifically on pH and solubility according to the International Standards Organization (ISO) 6876 method.

1.2 Problem Statement and Study Rationale

Due to its ability to reduce the remaining microorganisms in the root canal, medicated root canal sealer has drawn the attention of researchers. Zinc-oxide eugenol-based sealers, calcium hydroxide-based sealers, glass ionomers, resins, silicone sealers and sealer that contain pharmaceutical materials like Endomethasone are the examples of medicated root canal sealer that had existed in the market for long. Researchers continue to make efforts in enhancing the antibacterial properties of commercial root canal sealers by mixing in antibiotics. Sharma *et al.*, (2014) in their study incorporated different antibiotic types, including amoxicillin, metronidazole, azithromyacin, gatifloxacin and doxycycline into Kerr sealer EWT, Endomethasone, AH26, AH Plus and Roekoseal. It was demonstrated that the sealer-antibiotic combination containing amoxicillin had the significant difference compared to other combinations in regard of the antibacterial effects. Vanapatla *et al.,* (2016) suggesting that mixing a triple antibiotic mixture with zinc oxide eugenol is the most effective in inhibiting bacterial growth.Antibacterial properties are the goal in producing a novel endodontic sealer, yet we should not deny the threat, antibiotic resistance which can cause a global health crisis if we use them inappropriately (Bolfoni *et al.*, 2018). Therefore, it is important to conduct further research for alternatives that can serve the same purpose.

Recently, the use of herbal extracts has been emerging in endodontics. These will help reduce the cost, increase availability, increase shelf life, lower the toxicity, prevent microbial resistance, better tolerated by patients, and be renewable (Philip *et al.*, 2021). There have been efforts made to incorporate PB into dentistry, especially in the endodontic field due to its antibacterial properties against *E. faecalis* (Armianty and Mattulada, 2014; Cecilia *et al.*, 2016a; Pasril and Yuliasanti, 2014). Hence, this current study will incorporate PB into two different types of sealers since there is no research on natural herbal extract in the sealer, evaluating the pH value, solubility, cytotoxicity and antibacterial properties.

The outcome of this study is to enhance the effectiveness of commercialised sealers that are available nowadays and bring new perspectives /new options of medicated type root canal sealer.

1.3 Research Questions

1. Are there any differences in the pH between ethanolic extract of *Piper betle* in combination with AH Plus and BioRoot RCS and sealers alone?
2. Are there any differences in the solubility between ethanolic extract of *Piper betle* in combination with AH Plus and BioRoot RCS and sealers alone?
3. What is the difference in the viability of human periodontal ligament fibroblasts between ethanolic extract of *Piper betle* in combination with AH Plus and BioRoot RCS and sealers alone?
4. What are the differences in the antibacterial properties towards *E. faecalis* between ethanolic extract of *Piper betle* in combination with AH Plus and BioRoot RCS and sealers alone?

1.4 Objectives

1.4.1 General Objective

To investigate physicochemical properties (pH and solubility), cytotoxicity, and antibacterial effects of PB extract combined with AH Plus and BioRoot RCS.

1.4.2 Specific Objectives

1. To compare the pH of ethanolic extract of *Piper betle* in combination with AH Plus and BioRoot RCS and sealers alone.
2. To compare the solubility of ethanolic extract of *Piper betle* in combination with AH Plus and BioRoot RCS and sealers alone.
3. To determine and compare the cytotoxicity of ethanolic extract of *Piper betle* in combination with AH Plus and BioRoot RCS and sealers alone on human periodontal ligament fibroblasts.

4. To determine and compare antibacterial property of ethanolic extract of *Piper betle* in combination with AH Plus and BioRoot RCS and sealers alone against *Enterococcus faecalis* using modified direct contact test.

1.5 Research Hypothesis

1. There is no difference in pH between ethanolic extract of *Piper betle* in combination with AH Plus and BioRoot RCS and sealers alone.
2. There is no difference in solubility between ethanolic extract of *Piper betle* in combination with AH Plus and BioRoot RCS and sealers alone.
3. Ethanolic extract of *Piper betle* in combination with AH Plus and BioRoot RCS and sealers alone does not exhibit any cytotoxic effect on human periodontal ligament fibroblasts and has no difference between groups.
4. Ethanolic extract of *Piper betle* in combination with AH Plus and BioRoot RCS and sealers alone does not exhibit enhanced antibacterial activity against *Enterococcus faecalis* and has no difference among between groups.

CHAPTER 2

LITERATURE REVIEW

2.1 Microbes in Endodontic Infection

An endodontic infection has always been associated with microbes. Culture studies revealed that *E. faecalis* (Rôças *et al.*, 2004) and *C. albicans* (Siqueira Jr and Sen, 2004) are occasionally isolated from primary endodontic infections but more common in the obturated root canals that have failed (Siqueira JR and Rôças, 2014). The *E. faecalis* and *C. albicans* are resistant species in infected root canals, causing treatment failures (Bhayya *et al.*, 2021). A study suggested that the *E. faecalis* can be eliminated easily when they are present in small amount but not when they present in large amount (Narayanan and Vaishnavi, 2010). Root canal treatment aims to remove the microorganism and prevent reinfection, maximizing the endodontic treatment's prognosis via chemo mechanical procedure. However, with the complexity of root canal system and the limitation of instruments, it is virtually impossible (Siqueira JR and Rôças, 2014). Determining the need of eradicating bacteria to complete a successful root canal treatment is the foundation of the current study on the obturation material combine with natural product with antibacterial effects.

2.1.1 *Enterococcus faecalis*

E. faecalis is recognised as the most involved species from root canals that underwent retreatment, cases of failed root canal treatment (RCT), and cases of recalcitrant infections. *E. faecalis* are facultative anaerobes and gram-positive cocci, usually inhabit in the oral cavity and gingival sulcus. It is easier to be eliminated when they are in small numbers but not in significant numbers. Additionally, they have unique features which will help them to linger in the root canal system.

Culture and Polymerase Chain Reaction (PCR) techniques are the methods that are commonly used for the *E. faecalis* identification in endodontic infections (Baumgartner *et al.*, 2004). By using culture method, only 4 to 12.5% of primary endodontic infection and 18.5-20% of failed root canal treatments had *E. faecalis* present. In contrast, the percentage of *E. faecalis* involvement are higher via PCR method, 67 to 89.6% for primary endodontic infection and 33 to 89.3% for recurrent endodontic infection, respectively (Lins *et al.*, 2013). In several other research, *E. faecalis* was found to be associated with either primary (4-40%) or 2° endodontic infections (24–77% in some cases) (Ferreira *et al.*, 2015; Murad *et al.*, 2014; Ozbek *et al.*, 2009; Rôças *et al.*, 2004; Stuart *et al.*, 2006; Tennert *et al.*, 2014). *E. faecalis* is one most often isolated bacteria from patients of unsuccessful endodontic treatment (Murad *et al.*, 2014; Ozbek *et al.*, 2009; Pourhajibagher *et al.*, 2017). *E. faecalis* was found in 74.4% of root canal treated teeth compared to only 25% of 1° endodontic infections (Ozbek *et al.*, 2009). Another study that included the Turkish population and used the real-time PCR methodology, biochemical testing, and the RNA gene sequencing approach likewise verified this pattern of results (Pourhajibagher *et al.*, 2017). The success of RCT mainly depends on the elimination of microorganisms; hence it is imperative to understand the involved microorganism regarding its nature and pathophysiology.

2.1.1 (a) Survival of *E. faecalis's*

Multiple antibiotic intracanal medications such as tetracycline, metronidazole, erythromycin, clindamycin, ciprofloxacin, minocycline, chlorhexidine, clindamycin, gentamycin, rifampicin, and vancomycin have been reported to be ineffective against *E. faecalis* according to in-vitro and in-vivo research (Barbosa-Ribeiro *et al.*, 2016; Ferreira *et al.*, 2015). Biofilm formation, virulence factors, tolerance to high pH, and antibiotic resistance have been identified as possible causes for *E. faecalis* survival. They can resist the antibiotics that act on cell walls like ampicillin, penicillin and cephalosporin via altering the amino acid and protein sequences (Miller *et al.*, 2014; Rice *et al.*, 2004). Besides, with the aid of enterococcal enzymes or a mutation in the genes encoding nucleic acids, *E. faecalis* is also capable of changing the hydroxyl and amino group. This enables them to resist the antibiotic that interferes with the formation of protein (Miller *et al.*, 2014). The binding affinity of antibiotics that affect nucleic acid replication, transcription, and synthesis, such as quinolones, rifampicin, and trimethoprim, can be altered through mutations in the target genes (López-García *et al.*, 2020; Miller *et al.*, 2014).

E. faecalis can survive in the root canal system despite root canal therapy because the components that contribute to the virulence of it can help in host cell adherence, aid in tissue invasion, immunological regulation, and toxin release that damages host cells (Mishra *et al.*, 2017; Zou and Shankar, 2016). The factors include toxins such as hemolysin, cytolysin, gelatinase, and aggregation compounds, as well as cell wall polysaccharides, pheromones, and lipoteichoic acids. Enterococcal surface protein (ESP) is thought to aid bacterial persistence and colonisation during infection by forming biofilms, maintaining the pathogen's primary contact with the host surface, and utilising uroplakin or mucin to encourage bacterial and cell adherence to the host (Zoletti *et al.*, 2011; Zou and Shankar, 2016). In contrast to cytolysin, which causes cell lysis, hemolysin is a toxin that promotes the cell death of human erythrocytes and the infection to spread . Additionally, gelatinase induces the production of a collagen-binding protein (Mishra *et al.*, 2017), *E. faecalis* adherence to dentine, and encourages the development of biofilms (Rafi, 2022). Material for aggregation helps *E. faecalis* to adhere to host by attaching to the host's collagen and fostering the development of an antibiotic-resistant biofilm (Kafil and Mobarez, 2015). Additionally, aggregation chemicals increase the hydrophobicity of the cell surface and shield the cell from phagocytosis. It has been demonstrated to prolong phagocytosed *E. faecalis* 's intracellular survival (Halkai *et al*., 2012). These virulence traits together support *E. faecalis* remained alive and colonised the root canal.

Furthermore, *E. faecalis* could endure because of its capacity to stay alive in a situation despite pH changes. According to earlier research, it could survive in a pH range of 9.5 to 11.5 (Evans *et al.*, 2002). When negative hydroxyl ions reach the cytoplasm of bacteria, proton pump that pumps positive potassium ions into cell produces a low pH environment. In addition, to assist the cell survive the high pH, a change in the hydrophobicity of the cell surface and an upsurge in Na+ K+ -ATPase activity occurred (Ran *et al.*, 2013).

Other factors that promote *E. faecalis* survival is the formation of biofilm. They can grow on surfaces of biofilm and develop antibacterial resistance by preventing host defense and poisonous compounds from host. Additionally, the biofilm's carbohydrate/polysaccharide matrix serves as a physical barrier between *E. faecalis* and the surrounding environment (Flemming *et al.*, 2016). Moreover, biofilms assist bacterial species in nutrient intake, enabling them to survive in adverse environments (Simain et al., 2010).

2.2 Root Canal Sealers

A root canal sealer is used along with the core root canal filling material at the last stage of RCT. The main role of the sealer is to fill the gap between the root canal wall and the core material. A hermetic seal is created, and bacteria will be entombed, from entering the periapical tissue and entering the oral environment.

2.2.1 Ideal Properties of Root Canal Sealer

A root canal sealer should ideally have these qualities. Excellent root canal sealer properties include excellent adhesion to root canal walls, the capacity to create a tight seal, no shrinkage during setting, insolubility in tissue fluid, dimensional stability, biocompatibility, ease of handling and mixing, non-tooth discoloration, antibacterial properties, an acceptable setting time, and ease of removal during retreatment (Lin *et al.*, 2020). Insolubility properties significantly impact the success of the RCT as dissolution might cause gapping, which will lead to a bacteria ingression (Ørstavik *et al.*, 2001). "ANSI/ ADA specification No. 57 and International Standards Organization 6876 standard stated the requirement of insolubility for root canal sealer where the solubility of a sealer shall not exceed 3% mass fraction after immersion in water for 24 hours" (Poggio *et al.*, 2017a; Poggio *et al.*, 2007). Additionally, it is preferred that the sealer's pH value be alkaline as a high pH value results in an antibacterial properties that further lowers the residual infectious root canal bacteria, including *E. faecalis*, when contact with water (Prabhakar *et al.*, 2012) that survived the chemo-mechanical instrumentation. Furthermore, it was found that high alkalinity will improve the healing of periapical tissue by inhibiting osteoclast activity and promoting the apical root region's mineralised tissue deposition (Lin *et al.*, 2020).

However, none of the available root canal sealers in the market nowadays fulfil the ideal criteria.

2.2.2 Groups of Root Canal Sealer

Root canal sealers are divided into eight groups depending on their content, zinc oxide-eugenol-based, salicylate-based, resin-based, glass ionomer-based, and silicone-based sealers (Komabayashi *et al.*, 2020).

2.2.2 (a) Zinc oxide-eugenol-based sealer (ZOE)

ZOE sealers have been a standard in endodontics since they were created based on their long-term performance. It includes eugenol liquid, an essential oil made from cloves (Araki *et al.*, 1994; Fujisawa and Murakami, 2016), and zinc oxide powder to create an amorphous gel when applied to moist root dentine (Wilson and Mesley, 1972). Although certain powder-liquid sealers contain silver in the powder portion, this has led to tooth discoloration. As a result, formulations without silver were created to address the discoloration problem. ZOE is still a preferred option because of its slow setting time, low cost, antibacterial properties, and simplicity of use (Civjan and Brauer, 1964). Calciobiotic Root Canal Sealer (CRCS, Coltene/Whaledent, Switzerland) (ZOE sealer marketed as calcium hydroxide sealer), and Bioseal (OGNA. Pharmaceuticals, Muggiò, Italy) (a ZOE sealer with added hydroxyapatite) are examples of sealers with medicinal ingredients that frequently uses ZOE sealers as a matrix (Komabayashi *et al.*, 2020).

2.2.2 (b) Salicylate/ Calcium hydroxide-based sealer

This type of sealer is often sold and referenced to by its medicinal ingredients rather than its actual composition. Examples of calcium hydroxide-containing salicylate sealers include Sealapex^{TM} (Kerr, USA) and Apexit/Apexit® Plus (Ivoclar Vivadent, Schaan, Liechtenstein). Alkalinity and antibacterial capabilities in calcium hydroxide are both important traits in a therapeutic sealant (Staehle *et al*., 1995). However, due to its solubility, calcium hydroxide itself cannot be the sealer. A matrix must therefore contain them and serve as a reliable sealer (Cox and Suzuki, 1994). A similar salicylate resin-based sealer, MTA Fillapex (Angelus, Brazilian), should not be classified as a tricalcium silicate sealer because it is primarily made of resin and contains around 15% of the mineral trioxide aggregate (MTA) powder.

2.2.2 (c) Fatty acid-based sealers

Non-eugenol zinc oxide sealers were created to prevent problems with post-operative healing because of eugenol's cytotoxic effects (LINDQVIST and OTTESKOG, 1980). Although the structure of their metal complexes, eugenolates and salicylates, is frequently less defined and consistent by their nature, fatty acids are used as chelating agents instead of eugenol. For instance, Canals-N and Nogenol (Showa Yakuhin Kako Co., Ltd., Tokyo, Japan) (GC America, Alsip, IL, USA).

2.2.2 (d) Glass ionomer-based sealers

A fine silicate glass powder is mixed with polyacrylic and related acids to create glass ionomer sealer products. They combine to produce ionomers, which are repeating subunits of organic monomers and inorganic ions (Berg and Croll, 2015). In dentistry, these materials are used for cement and restoratives. KT-308 (GC, Tokyo, Japan) is a glass ionomer cement sealer that emits fluoride to prevent decay and adhere to the tooth structure (Ogasawara *et al.*, 2003), but it is no longer commercially available. Glass ionomer sealer Ketac-Endo (3M ESPE, St. Paul, MN, USA) is accessible in some parts of the world.

2.2.2 (e) Silicone-based sealers

Similar to epoxy resin-based sealants, silicone-based sealants also polymerise through an addition reaction. By adding polymerisation as a sequence of cross-linkages between divinylpolysiloxane and polymethylhydrosiloxane with a platinum salt as the catalyst, silicone-based sealers create a three-dimensional polymer network. Examples of silicone-based sealers include GuttaFlow, GuttaFlow 2, and RoekoSeal from Coltene/Whaledent (Zhou *et al.*, 2013). Unlike GuttaFlow 2 and RoekoSeal, which are auto-mix systems, GuttaFlow is triturator-mixed and calls for the use of a single master cone.

2.2.2 (f) Epoxy resin-based sealers

Epoxy resin-based sealants, such as AH 26 and AH (Dentsply Sirona, Konstanz, Germany), are composed of amines and low-molecular-weight epoxy resins. They are set by an addition reaction between the epoxide groups attached to the epoxy resins and the amines, producing a polymer. While AH is composed of paste and paste, AH 26 is composed of powder and paste. When AH is offered in an automatic mixing syringe, it is branded as “AH Jet”. ThermaSeal Plus and Ribbon sealer are a couple of the different names that AH and AH Jet are offered as in the US. AH is sometimes referred to as “TopSeal” in Central America, South America, and

Europe. Regarding the physicochemical characteristics of a sealer for root canal filling, AH is regarded as the gold standard (Zhou *et al.*, 2013).

2.2.2 (g) Tricalcium silicate-based (MTA/bioceramic) sealers

MTA is a ceramic cement made from dicalcium silicate and tricalcium silicate hydraulic granules. Compared to ceramic powders with the same ceramic phases found in Portland cement (Torabinejad and White, 1995), it is a more pure and finer powder with some radiopaque excipients. Both calcium hydroxide and calcium silicate cement are bioactive, releasing calcium and hydroxide ions when used in ceramics (Gandolfi *et al.*, 2015). The ions cause the surface of body fluids (or artificial body fluids) to create hydroxyapatite when they are present.

ProRoot MTA Gray (Dentsply Sirona, Johnson City, TN, USA), the first well-known MTA product, has been available since 1997 and has solely been utilised as a root-end filler material or perforation fill. A commercially available salicylate resin-based sealer called MTA Fillepex (Angelus, Brazilian) that contains 15% MTA powder was introduced in 2010. Tricalcium silicate compounds have the advantages of biocompatibility and sealing through the development of hydroxyapatite (Darvell and Wu, 2011). Granules of tri- and dicalcium silicate react with water to form a hydrated calcium hydroxide matrix. The calcium and hydroxide ions keep releasing after settling for roughly a month (Gandolfi *et al.*, 2015). Due to the elevated pH, the phosphate ions in body fluids precipitate hydroxyapatite close to the surface (Komabayashi *et al.*, 2016). Apatite-like crystals have been reported to form in the apical and middle thirds of canal walls after using tricalcium silicate-based sealers. Other powder liquids commercial tricalcium silicate sealers have been created since the release of the MTA Plus product like BioRoot RCS (Septodont, France) and Endo CPM Sealer (EGEO, Buenos Aires, Argentina).

Additionally, single-component and two-paste materials are commercially available, however physicians prefer the single-paste tricalcium silicate-based sealers due to their simplicity of usage despite their high price. The development of hydroxyapatite at the surface of the canals in conjunction with water absorption from dentin tubules is the mechanism by which single-paste tricalcium silicate-based sealers set. In a non-surgical root canal procedure, the EndoSequence BC Sealer (Brasseler, USA) is employed with a single-cone approach as a viable obturation alternative (Chybowski *et al.*, 2018). Tricalcium silicate materials have been marketed as "bioceramics" or "biosilicates", but these words are overly general since many dental materials are bioceramics (Komabayashi *et al.*, 2016). The materials made of tricalcium silicate are unique in their bioactivity or ability to perform biological functions that produce hydroxyapatite on their surface and have an osteogenic effect (Faraco Jr and Holland, 2001).

2.2.2 (h) Methacrylate resin-based sealers

Methacrylate resin-based sealers are divided into several generations. When dentinal bonding was still in its infancy, the first generation of methacrylate-based sealers, Hydron, made its appearance in the middle of the 1970s (Kronman *et al.*, 1979). In the 1990s, the second generation of bondable sealers was unveiled. It is hydrophilic, non-etching, and does not require the application of a dentine adhesive. To make bonding processes simpler, new generations of self-etching (third generation) and adhesives have been created. In terms of the lack of a separate etching/bonding step, the fourth-generation methacrylate resin-based sealers (such as MetaSEAL, Parkell Inc.; RealSeal SE, SybronEndo) are functionally analogous to a class of interchangeable self-adhesive resin luting composites (Radovic *et al.*, 2008).

2.2.3 Antibacterial Properties of Root Canal Sealer

A root canal sealer can either directly or indirectly cause antibacterial activity by entombing bacteria in a hermetic seal that prevents residual bacteria from communicating with the apical tissue. However, bacteria at the apex will not be entombed and will only be killed by an antibacterial endodontic sealer (Komabayashi *et al.*, 2020). Previous root canal sealers have shown limited bactericidal activity over time, which is mediated by chemicals released during the setting process. Among the root canal sealers, epoxy resin-based sealers present short-term antibacterial effects due to the formaldehyde or bisphenol A diglycidyl ether released during their setting process (Slutzky-Goldberg *et al.*, 2008; Zubizarreta-Macho *et al.*, 2021). Similar to silicone-based and calcium hydroxide-based sealants, which have a moderate antibacterial effect, zinc oxide eugenol-based sealers produce eugenol particles during the curing stage. (Kapralos *et al.*, 2018; Singh *et al.*, 2016). On the other hand, bioceramic sealers made of calcium silicate and phosphate and MTA-based sealers have significant antibacterial properties due to the high pH and the compounds and ions produced during the setting (Singh *et al.*, 2016). Besides, research on medicated type root canal sealer and the development of nanotechnology-based endodontic sealer with quaternary ammonium polyethylenimine nanoparticles in them showed a significant improvement in antibacterial properties against *E. faecalis* strains (Kesler Shvero *et al.*, 2016).

According to Nawal *et al.*, (2011), Epiphany had the strongest bactericidal effects among the sealers, followed by AH-Plus sealer and Guttaflow. For epoxy resin-based sealer, the antibacterial effect of AH is less when compared to AH 26 due to the lack of formaldehyde release, which is cytotoxic but still effective in reducing the number of cultivable cells of *E. faecalis* (Subbiya *et al.*, 2021). Similarly, AH sealer has better antibacterial action when compared to GuttaFlow. However, it was less antibacterial than MTA Fillapex (salicylate-based sealer) and CRCS (ZOE-based-sealer) (Shakya *et al.*, 2016).

Root canal sealers with integrated calcium hydroxide have enhanced antibacterial activity. Apexit Plus is a calcium hydroxide–based material which contains salicylate, marketed as medicated root canal sealer. Research reported that Apexit Plus exhibited higher antibacterial activity and was more effective against the tested microorganism than the other materials AH, Epiphany SE, and RoekoSeal (Slutzky-Goldberg *et al.*, 2008). As for tricalcium silicate-based sealers/cement (EndoSequence BC Sealer and ProRoot MTA), they had higher antibacterial activity for *E. faecalis* than both epoxy resin (AH) and ZOE-based-sealers (Singh *et al.*, 2016; Wainstein *et al.*, 2016) due to calcium and hydroxide ions are released, resulting in a high pH.. (Duarte *et al.*, 2003).

Besides, the development of bioceramic-based sealer has demonstrated to enhance the effectiveness of root canal therapy with its properties such as hydroxyapatite formation chemical stability, biocompatibility, flowability, calcium ion release, hydrophilicity, and biomineralization (Moraes *et al.*, 2022), which contribute to a hermetic seal of the root canal therapy (Bel Haj Salah *et al.*, 2021). In a study done by Kharouf *et al.*, (2020) using a direct contact test, the BR sealer had a higher antibacterial capacity than CeraSeal after 24 hours. Similar findings were observed in a recent study (Abduljabbar and Abumostafa, 2021). Another study reported that the antibacterial effects of BR was significantly higher than that of other sealers (Totalfill BC and AH sealers) after 30 days of exposure toward the material (Alsubait *et al.*, 2019). BR was also reported to have a significantly higher antibacterial activity than AH after seven days of exposure (Arias-Moliz and Camilleri, 2016).

2.2.4 Natural Products in Root Canal Sealer

In the past decades, research on the use of medicinal herbs as antibacterial therapy has grown. This approach is of worldwide interest as it is sustainable and serves as an alternative strategy to antibiotics, which will cause antibiotic resistance. In dentistry, antibiotics were mostly used to treat diseases related to the root canal system (AboAlSamh *et al.*, 2018). It was extensively prescribed not only systemically but also locally, such as the use of intracanal medicament, endodontic sealer with antibiotics, medicated gutta percha, and more (AboAlSamh *et al.*, 2018). Studies show dental practitioner still prescribe antibiotic in nonindicated conditions even they are aware of antibiotic resistance (Bolfoni *et al.*, 2018; Segura-Egea *et al.*, 2017).

Hence, incorporation of natural product can be seen in a different stage of RCT, such as cleaning and irrigation, sealer cement to lubricate and aid in bonding of gutta-percha (GP), removal of GP through softening and dissolving it, removal of smear layer, storage media for avulsed teeth, and pulp and dentine repair (Almadi and Almohaimede, 2018). When a natural product, plants or herbs are mixed with root canal sealer, it should be radiopaque, not staining the tooth, stable in dimension, easily mixed and applied into the canal, bacteriostatic and not cytotoxic to periradicular tissues (Yadav, 2021).

In research, the calcium silicate sealer was modified by adding hinokitiol, a naturally occurring substance found in the wood of trees of the Cupressaceae family showed suitable setting time and solubility, antibacterial synergistic effect and active ability of odontoblastic differentiation of human dental pulp cells (hDPCs) (Huang *et al.*, 2016). Experimental resin-based root canal sealers containing butia or copaiba natural oils demonstrate similar film thicknesses, flow values, adequate cell compatibility and enhanced antibacterial effect compared to commercial methacrylate-based resin material (Reiznautt *et al.*, 2021).

Besides, root canal sealers incorporated with extracts from *Ricinus communis* (castor oil polymer) and *Copaifera multijuga* as natural resins were also reported to be biocompatible and not cytotoxic at any concentration when compared to synthetic resin-based sealers (Silva *et al.*, 2016b). Another study incorporating *Copaifera multijuga* oil as natural resin in root canal sealer concluded that this sealer had satisfactory results in the physicochemical tests according to ADA standard (Garrido *et al.*, 2010). A study also showed that cinnamon oil is inherently antibacterial and can be used with root canal sealers without hindering the sealer's efficiency (Cinthura and Geetha, 2018). *Tagetes minuta* and *Mentha. piperita* essential oils and the crude ethanolic tincture of Bixa orellana vegetable extracts in root canal sealer also demonstrated antibacterial potential with excellent physical-mechanical properties (dos Santos *et al.*, 2021). For propolis, trans-trans farnesol is a terpenoid responsible for antibacterial activity. Its pharmacological characteristics, such as low cytotoxicity and genotoxicity, make it safe to use in dentistry. A recent study found that mixing the trans-trans farnesol into Sealapex sealer reduced *E. faecalis* growth *in vitro* when compared to just using Sealapex sealer itself (Diogo De-Carli *et al.*, 2021).

Natural products have a lot of potential in dentistry, particularly in endodontics, because of their antibacterial properties. Moreover, they are readily available, economical, have a longer shelf life, are highly biocompatible, and lack microbial resistance (Ambareen and Chinappa, 2014).

2.3 *Piper betle*

Piper betle (PB) is also known as betel vine (English) and 'sireh' (Malay). It is a medicinal plant that belongs to the family *Piperaceae*. Betel is mainly eaten as betel quid in Asia (and more recently by emigrants from these regions in other parts of the world). Betel-quid sometimes includes additional ingredients such as areca nut, tobacco, and spices (Azahar *et al.*, 2020). Phytochemical studies showed that PB contains a wide variety of biologically active compounds whose concentration depends on the variety of the plant, season and climate (Umar *et al.*, 2018).

2.3.1 Composition of *Piper betle*

The fresh betel leaves were found to consist of water (85-90%), protein (3-3.5%), fat (0.4-1%), carbohydrates (0.5-6.1%), fibers (2.3%), essential oil (0.08-0.2%) and tannin (0.1-1.3%) (Lakshmi *et al.*, 2005). (Guha, 2006) revealed that the leaves are packed with vitamins and minerals such as calcium, phosphorus, potassium, iron, iodine, carotene, nicotinic acid, thiamine, riboflavin, vitamin C and a significant amount of amino acids. In addition, a phytochemical analysis performed by Sugumaran *et al.*, (2011) showed that the betel leaves contained saponin, phenol, alkaloids, amino acids, tannins, flavonoids, steroids and other compounds.

Chemical constituents of the *Piper betle* include allyl pyrocatechol, chavibetol (53.1%), eugenol, quercetin, safrole, caryophyllene (3.71%), hydroxychavicol, camphene, chavibetol methyl ether, myrcene, a-pinene, chavicol, aterpineol, piper betol, (Nagori *et al.*, 2011; Shah *et al.*, 2016). Eugenol (11.92%), hydroxychavicol (66.55%), isoeugenol (2.90%), and 4-allyl1,2,diacetoxybenzene (3.21%) were the main phenols discovered in the *Piper betle* (Ali *et al.*, 2010). Hydroxychavicol has anti-inflammatory properties; eugenol and chavibetol have antibacterial, antioxidant, antiviral, analgesic, anticancer, and depressive properties; and antioxidative, antibacterial, and mucin properties (Sharma *et al.*, 2009).

2.3.2 Methods of Extraction

Maceration, percolation, and infusion are the general techniques used for the extraction of medicinal plants and are mainly applied for galenical preparations. The sole purpose of such basic extraction procedures is to obtain the therapeutically desirable portion and eliminate the inert material by treatment with a selective solvent known as the menstruum (Singh, 2008).

Pin (2010) evaluated the effects of solvents such as water, ethanol, ethyl acetate, and hexane on the extraction of *Piper betle*. Results showed that extraction yield was highest for water, followed by ethyl acetate, ethanol, and hexane. This provided evidence that phytochemicals of *Piper betle* have high polarity so are easily soluble in the water. Contrary to this report, phenolic content was found to be the greatest with ethanol compared to water in another (Ali *et al.*, 2018). Method of extraction can also influence the quality of extract, as the ultrasonic-assisted extraction was found to result in more flavonoid, phenol, and eugenol content compared to maceration and solvent extraction (Das *et al.*, 2019). However, ultrasonic-assisted extraction may result in the degradation of the phenolic components of the plants (Azwanida, 2015). In contrast to previous studies, (Taukoorah *et al.*, 2016) suggested that the phytochemicals present in PB by maceration technique had significant total flavonoid content (TFC) and total phenolic content (TPC). In conclusion, evidence from the limited literature indicates that ethanol is the best solvent and the maceration technique is the safest for extracting PB.

The total phenolic compound of an ethanolic extract of PB was almost three times that of an aqueous extract (Nouri, Mohammadi Nafchi and Karim, 2014). Ethanol was found to be the most effective in extracting phenolic compounds such as chavicol, hydroxychavicol, chavibetol, chavibetol acetate and eugenol (Azahar, Mokhtar and Arifin, 2020). As a result, the ethanolic extract of PB has demonstrated higher anti-microbial properties (Dwivedi and Tripathi, 2014). A study by A. Ali *et al.*, (2018) demonstrated that the ethanolic extract of PB incorporated into commercial toothpaste had a significant growth inhibitory effect on bacterial suspensions of *Escherichia coli, Staphylococcus aureus, Streptococcus mutans, Streptococcus salivarius* and a fungal suspension of *C. albicans.* This extract was also shown to have greater antioxidant and free radical scavenging properties (Shah *et al.*, 2016).

2.3.3 Antibacterial Properties of *Piper betle*

PB leaf extracts have previously been tested for antibacterial activities against bacterial and fungal pathogens. Punareewattana and Aiemsaard (2016) found that PB leaf extracts at 5% concentration exhibit antibacterial activity as 0.12% chlorhexidine against *Porphyromonas gingivalis*, a bacterium causing periodontitis. Another study found that 0.1% PB extract had antibacterial and antifungal activity against *E. faecalis and C. albicans* (Bhayya *et al.*, 2021).

Ethanol and aqueous extracts reported higher antibacterial activity against drug-resistant bacterial isolates, among other different solvent extracts of PB. The ethanolic extracts of PB showed the maximum zone of inhibition against *Escherichia coli*, which was slightly lower by 0.4 mm compared to the inhibition zone of the standard antibiotic imipenem. In comparison, petroleum ether and chloroform extracts of PB leaves did not profoundly affect test bacteria (Saranya and Anuradha, 2020). In a study done by Teanpaisan *et al.,* (2017), researchers reported a minimal inhibitory concentration of 0.521% concentrated ethanol extract of PB against *E. faecalis*, whereas onerecent study done by Rafi (2022) reported a slightly lower concentration of concentrated ethanol extract of PB, 0.312%.

PB extract was discovered to contain fatty acids (stearic acid and palmitic acid), hydroxy fatty acid esters (hydroxy esters of stearic acid, palmitic acid and myristic acids) and hydroxychavicol, with the latter as the main component. Hydroxychavicol is claimed to have antibacterial properties. Besides, fatty acids can behave as anionic surfactants. At low pH, they are antibacterial and antifungal in addition to being selective towards Gram-positive organisms by targeting the structure and function of bacterial cell walls and membranes (Nalina and Rahim, 2007).

2.3.4 Research on *Piper betle* in Dentistry

Numerous studies have been conducted and are ongoing to explore the potential of natural products incorporated in dental materials due to their antibacterial properties and biocompatibility. However, there is no study yet on PB combined with root canal sealer.

There have been studies done that show that PB is among the plants that have been associated with the control of periodontal disease and caries. The crude extract of PB leaves may exert anti-cariogenic activities related to decreased acid production and changes to the ultrastructure of *Streptococcus mutans* (Nalina and Rahim, 2007). It was also reported that aqueous extract of PB leaves inhibits adherence of early plaque settlers, including *Streptococcus mitis*, *Streptococcus sanguinis* and *Actinomyces* sp. to a saliva-coated glass surface (Razak and Abd Rahim, 2003). A previous study by Wardhana *et al.,* (2017) reported that the formulation of toothpaste