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Cytotoxic and Antibacterial Properties of *Piper betle* Combined with Selected Root Canal Sealers

(Sifat Sitotoksik dan Antibakteria *Piper betle* Digabungkan dengan Pengedap Saluran Akar Terpilih)

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ABSTRACT

Exploration in endodontic material had been made to maximise the elimination of microorganisms in root canal system to improve the treatment success rate. Inappropriate use of antibiotics can cause antibiotic resistance hence alternative is needed. *Piper betle* (PB), a medicinal herb to be used for its effective antibacterial properties against *Enterococcus faecalis.* Therefore, this study aims to investigate the 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 Plus (AH); PB and AH plus (PBAH); BioRoot RCS (BR); and PB and BioRoot RCS (PBBR). At 24, 48, and 72 h, 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, day1-set and day-7-set state of tested materials. Data were analysed with SPSS version 25 with Tukey's post-test at the level of significance of $p = 0.05$. PBAH showed significant lower cytotoxicity than AH ($p = 0.028$) at 48 h, with a higher percentage of cell viability compared AH (26.35%). PB reduce antibacterial effect of BR at all times at significant level ($p = 0.000, 0.014, 0.032$). Conclusion, PBAH has significant reduced cytotoxicity as compared to AH on HPdLF at 48 h. PBBR was cytotoxic to HPdLF at 24 and 48 h. 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.

Keywords: AH Plus; Bioroot RCS; natural product

ABSTRAK

Penerokaan dalam bahan endodontik telah dilakukan untuk memaksimumkan penyingkiran mikroorganisma dalam sistem saluran akar bagi meningkatkan kadar kejayaan rawatan. Penggunaan antibiotik secara tidak wajar boleh menyebabkan rintangan ketahanan terhadap antibiotik. Oleh itu, penggantian diperlukan. *Piper betle* (PB) ialah herba perubatan yang digunakan kerana keberkesanan sifat antibakterianya terhadap *Enterococcus faecalis*. Oleh itu, penyelidikan ini bertujuan untuk mengkaji kesan sitotoksik dan antibakteria ekstrak etanol PB dalam gabungan dengan AH Plus (AH) dan BioRoot RCS (BR) serta pengedap AH dan BR sahaja. Empat bahan yang diuji adalah; AH Plus (AH); PB dan AH plus (PBAH); BioRoot RCS (BR); serta PB dan BioRoot RCS (PBBR). Asai MTT ke atas HPdLF dijalankan untuk mengukur kesitotoksikan pada 24, 48 dan 72 jam. Kesan antibakteria dinilai dengan ujian sentuhan langsung diubah suai (MDCT) terhadap *E. faecalis* pada keadaan bahan yang diuji yang segar, set pada hari pertama dan set pada hari ketujuh. Data dianalisis menggunakan SPSS versi 25 dengan ujian pasca Tukey pada taraf keertian P = 0.05. PBAH menunjukkan sitotoksik yang lebih rendah secara signifikan berbanding AH ($P = 0.028$) pada 48 jam dengan peratusan kelangsungan sel yang lebih tinggi berbanding dengan AH (26.35%). PB mengurangkan kesan antibakteria BR pada semua masa pada tahap kepentingan yang signifikan ($P = 0.000, 0.014, 0.032$). Kesimpulannya, PBAH mempunyai pengurangan kesitotoksikan secara signifikan berbanding dengan AH terhadap HPdLF selepas 48 jam. PBBR menunjukkan kesan sitotoksik kepada HPdLF pada 24 dan 48 jam. Kedua-dua AH atau PBAH menunjukkan sifat antibakteria terhadap *E. faecalis*, diikuti oleh BP dan PBBR. Kesimpulannya, PBAH adalah pengedap kanal akar yang berpotensi ditingkatkan dan boleh dikaji lebih lanjut untuk mengenal pasti sepenuhnya sifatnya yang mendalam.

Kata kunci: AH plus; Bioroot RCS; produk semula jadi

Introduction

Cleaning and shaping, disinfection, and three-dimensional sealing of the root canal system are determinants in the effectiveness of endodontic therapy (Tomer et al. 2021). Complete sealing of the root canal system is crucial during obturation as it can prevent the reach of bacterial from oral environment to periapical tissue which cause the recurrent infection of root canal (Colombo et al. 2018; Sharad 2011). According to Khalifa et al. (2016), *Enterococcus faecalis* is notoriously difficult to eliminate and is responsible for repeated root canal treatment failures. Once *E. faecalis* has established itself in the dentinal tubules, it is very difficult to eradicate. Hence, a root canal sealer with antibacterial properties can continue the elimination of infectious microorganisms as the last medicament for root canal therapy (Sharma et al. 2014).

To overcome this issue, combination of antibiotics with sealer was used as the efficacy is significant, but it can cause antibacterial resistance if inappropriately used. In the market, there is tricalcium silicate-based sealer, a new type of sealer has the most superior antibacterial properties (Komabayashi et al. 2020), better than the AH Plus (Singh et al. 2016; Wainstein et al. 2016), the gold standard for endodontic sealer which is mostly used by all (Lee et al. 2017). However, tricalcium silicate-based sealers are expensive. Tricalcium silicate-based sealer antibacterial effect is due to the release of calcium and hydroxide ions, which cause the alkalinity whereas AH Plus's antibacterial effect due to the release of formaldehyde, epoxy, and amine content during the polymerization process (Nirupama et al. 2014). Even though AH is efficient in lowering the amount of *E. faecalis* cells that are cultivable, formaldehyde is poisonous to cells (Subbiya et al. 2020).

As herbal extract include unique bioactive components with antibacterial, anti- inflammatory, sedative, and anxiolytic actions, they are an alternative therapy method (Buggapati 2016), especially in endodontics for the antibacterial properties. Additionally, they are inexpensive, widely accessible, longer-lasting, extremely biocompatible, and microbial resistance-free (Zeenath & Chinappa 2014). The Piperaceae family includes the well-known medicinal plant *Piper betle* (PB), which is mostly found in South East Asia (Azahar, Nadzirah & Mohd Azmir 2020). According to a study by Bhayya et al. (2021), PB has potent antibacterial and antifungal actions against *Candida albicans* and *E. faecalis* when used as an irrigation solution. As of right now, PB has been added to mouthwash (Harshitha, Ramya & Bagavad 2020) and dental toothpaste (Ameena, Lim & Puteri 2018). When added to toothpaste, PB extract exhibits promising antibacterial properties; when added to mouthwash, PB extract exhibits effective anti-inflammatory, anti- plaque properties; thus, it is helpful in supportive periodontal therapy. However, there has not been any research on PB in combination with root canal sealant. Determining the cytotoxicity and antibacterial effects of PB extract in combination with AH Plus and BioRoot RCS was the goal of the current investigation. The hypothesis tested was that ethanolic extract of PB in combination with AH and BR and sealers alone does not exhibit any cytotoxic effect on human periodontal ligament fibroblasts and does not exhibit enhanced antibacterial activity in comparison with the PB extract alone against *E. faecalis*.

Materials and Methods

In Kubang Kerian, Kelantan, fresh PB leaves were acquired from a neighborhood garden. Plant samples were taken to the Herbarium Unit, School of Biological Sciences, USM, Penang, for identification, authentication, and deposition of voucher specimens (Herbarium No: 11877). In this investigation, AH Plus (Dentsply De Trey Gmbh, Konstanz, Germany) and BioRoot RCS (Septodont, France) root canal sealers were used.

A total of four groups were evaluated for antibacterial and cytotoxicity as follows:

1. Group AH : AH Plus
2. Group PBAH : PB + AH Plus
3. Group BR : BioRoot RCS
4. Group PBBR : PB + BioRoot RCS

A group PB (PB only) evaluated along with the four groups for cytotoxicity test only.

Extraction of EEPB

Five hundred grams of PB leaves were cleaned and dried for 5-7 days at 27 °C in an incubator. A lower particle size offers a greater surface area for solvent interactions, hence the sample was next processed using an electric grinder (Azwanida 2015). The PB ethanolic extract was prepared as described by Nair et al. (2008), except for the time parameters. The maceration technique was used

in this research, where ground materials were soaked in a solvent in a stoppered container and allowed to stand at room temperature for at least three days while being continuously stirred (Azwanida 2015). One hundred mL of 70% ethanol was used to extract 10 g of the powdered substance over the period of 72 h. Following that, it was centrifuged for 15 min at 5000 rpm after being filtered with Whatman filter paper No. 1. Upon collecting the supernatant, the solvent was evaporated and kept at 4 °C.

PREPARATION OF EEPB

Fifty mg/mL concentrations of EEPB were prepared. For cytotoxicity testing, 50 mg/mL was chosen since a study was conducted with the same batch of PB leaves shown that this concentration was not hazardous to HPdLF (Rafi 2021). A preliminary investigation was conducted to establish the ethanolic extract of PB's minimum inhibitory concentration (MIC) for *E. faecalis*. The following concentrations were used: 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 mg/mL. At 50 mg/mL, a colour shift was seen, hence that was used as the MIC value. It was subsequently chosen to be included in the sealer combination for MDCT in Group PBAH and Group PBBR.

PREPARATION OF TESTED MATERIALS IN EACH GROUP

The preparation of samples was done as follows. The concentration of EEPB used was 50 mg/mL. For Group AH, AH Plus was mixed following the manufacturer's recommendations until consistent colour was achieved. Paste A and Paste B were combined in equal volume units on a mixing pad with a spatula until a homogenous consistency was seen. In regards of Group PBAH, the same procedure was used, and later followed by the addition of 25 µL mixture of 50 mg/mL of EEPB. The mixture was then combined to create a consistent colour (Sharad 2011).

For Group BR, BioRoot RCS was mixed according to the manufacturer's recommendations until a uniform colour was obtained as follows: A level spoonful of powder was placed on mixing pad, 5 drops of mixing solution from single dose container were poured on the mixing pad. Mix must be made extemporaneously until a smooth paste (about 60 s) was obtained. Do not incorporate any remaining powder into the liquid as soon as a smooth, creamy consistency was obtained. Meanwhile, for Group PBBR, 25 µL mixture of 50 mg/mL of EEPB was combined into the mixture of BR, with sterile micropipette. The mixture was then combined to create a consistent colour.

MTT ASSAY ON HUMAN PERIODONTAL LIGAMENT FIBROBLAST (HPdLF)

HPdLF was taken out from a tank of liquid nitrogen and subsequently revived. It was then grown in Dulbecco's Modified Eagle's medium (DMEM) (Gibco, USA) as a basal medium, supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), and antibiotics (100 units mL^{-1} penicillin, 100 g mL^{-1} streptomycin), incubated in the incubator with 5% CO_2 at 37 °C temperature. This medium was changed every other day until confluence of 80% was reached. HPdLF were used in the 3rd passage. The cells were detached, counted, and seeded onto 96-well plates at a density of 3500 cells per cm^2 prior to experiments.

The EEPB was diluted in DMEM supplemented with 10% FBS and 1% Penicillin- Streptomycin to attain 50 mg/mL in order to produce the sealer specimens and extract. Then, materials (AH and BR) were mixed according to the manufacturer's instructions. 25 µL of PB extract at a concentration of 50 mg/mL were added to the materials, obtaining PBAH, and PBBR. All Group AH, PBAH, BR, and PBBR specimens were placed within a silicone mould that was made to generate discs that were 5 mm in diameter and 3 mm high. Materials were allowed set for 24 h under aseptic conditions and a humid environment. Each specimen was placed into 1 mL of DMEM supplemented with 10% FBS after setting, and it was then let to incubate for 72 h (Karimjee et al. 2006). The samples were subsequently discarded, and the eluate extracts were filtered through membranes with a 0.22 µm pore size (Millipore; Billerica, MA, USA).

One hundred µL of cell suspension at 3500 cells/cm^2 were plated in 96-well plates and later incubated with 100 µL of specimen medium in octuplicate. Supernatant was collected after 24, 48, and 72 h. Later, the cells were subject to the (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) assay for cytotoxicity assessment. Negative controls were performed in empty (not cell-containing) wells.

After the incubation time, 10 µL of an MTT solution (Sigma-Aldrich, St. Louis, MO, USA) (5 mg/mL) in phosphate buffered saline (PBS) was added to the wells. Fibroblasts were incubated at 37 °C in a dark environment for 4 h. One hundred µL of DMSO was added in lieu of all solutions. For 15 min, the plate was continuously agitated. The optical densities were measured at 570 nm in a spectrophotometer (FLUOstar Optima; BMG Labtech, Ortenberg, Germany).

ANTIBACTERIAL ACTIVITY AGAINST *E. faecalis*

Retrieval of *E. faecalis* was done using loop from the bacterial stock, and was cultured on BHI agar (Oxoid, UK), later incubating at 37 °C, anaerobic overnight. The next day, a sterile cotton swab was used to transfer 2 to 3 bacterial colonies into 5 mL of prepared BHI broth. Later, densitometer was used to measure the bacterial suspension and adjust it to the 0.5 McFarland standard (equal to 1.5×10^8 CFU/mL) after an overnight incubation at 37 °C.

The MDCT was carried out in line with Huang et al.'s (2019) instructions. Using the Dycal applicator, test materials were coated at the specified area (5 mm diameter and 1 mm thickness) on the edge of a well in a 96-well microtiter plate that was positioned vertically as shown in Figure 1. Materials were incubated anaerobically at 37 °C in > 95% humidity for 30 min (fresh), 24 h, and 168 h before being exposed to bacteria in order to assess the material at various times of setting. The material was covered with 10 µL of bacterial suspension at each time interval. Uncoated wells served as the positive control, while the material that was incubated without the bacterial suspension served as the negative control. All the samples were then incubated anaerobically for a further hour at 37 °C with a humidity of greater than 95%.

Then, each well received 190 µL of sterile saline, which was carefully mixed in using a pipette. In sterile saline, 10-fold serial dilutions were performed, and then were plated on agar. After a 24 h period of anaerobic incubation at 37 °C, the CFUs were counted, and the CFU/mL was computed. The tests were carried out three times. The MDCT procedures are shown schematically in Figure 2.

Using SPSS version 25 (IBM Corp., Armonk, NY, USA), all statistical analysis was carried out using analysis of variance (ANOVA) and the post hoc test at the level of significance of $P = 0.05$.

RESULTS AND DISCUSSION

CYTOTOXICITY

Figure 3 displays the results of the MTT experiment throughout the time intervals. The optical density (OD) and the percentage of cell viability (PCV) were often inversely proportional. The percentage of viable cells increases with increasing OD value, and vice versa. To comprehend the time-dependent toxicity of the tested materials, the PCV for the materials was obtained at 24, 48, and 72 h. The International Organisation for Standardisation (ISO), which classifies cell viability below 70% as cytotoxic [ISO:2009], is used to assess the results.

The HPdLF treated to complete medium showed a significant rising trend ($p < 0.05$) over the course of 24 to 72 h; this group is known as the control group. The

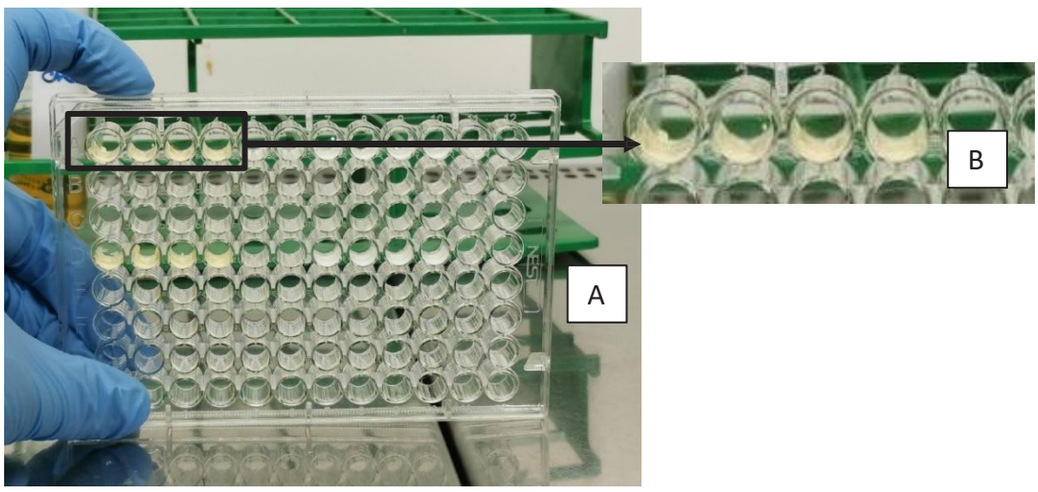

FIGURE 1. Samples were coated at the fixed area on the side of a well in a vertically positioned 96-well microtiter plate. B showing an enlarged image of A

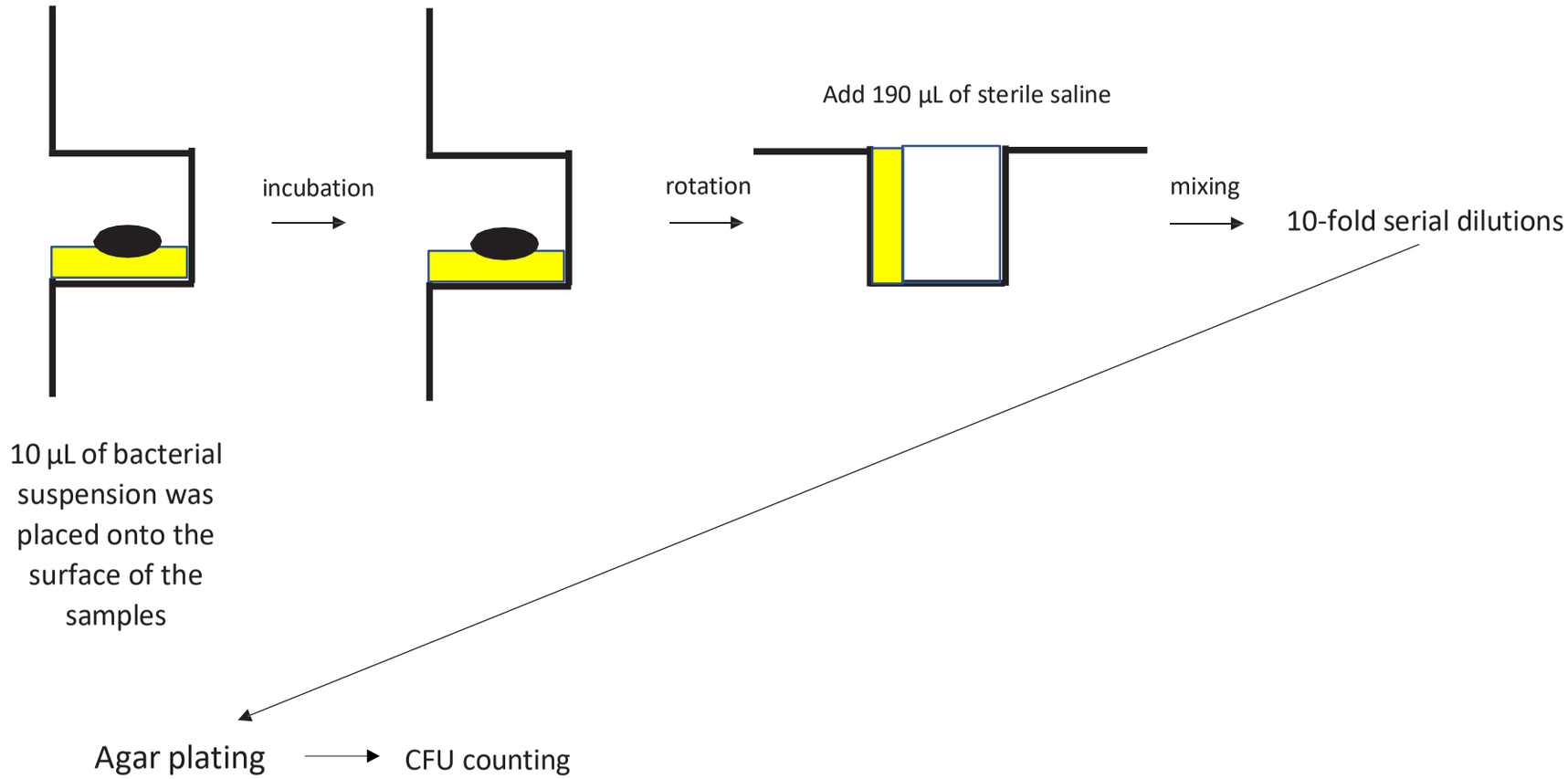

FIGURE 2. Schematic diagram of MDCT

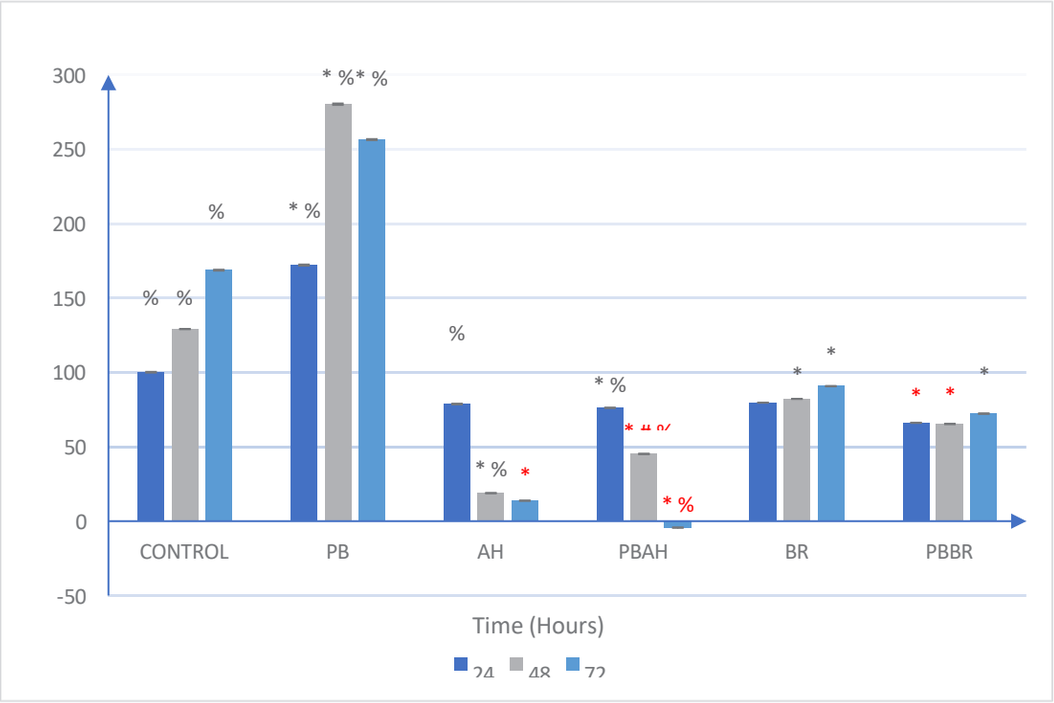

FIGURE 3. Cell viability of HPdLF treated with the test media for 24, 48 and 72 h using MTT assay

Red figures: cell viability below 70% as cytotoxic, International Organization for Standardization (ISO) [ISO:2009].

* : Statistically significant differences compared to the control group at a different time intervals ($p < 0.05$)

: Statistically significant differences observed in comparison among a commercial group with experimental group ($p < 0.05$)

% : Statistically significant differences observed when comparing among the group across the time interval ($p < 0.05$)

percentage of viable cells in the PB eluate significantly increases ($p < 0.05$), whereas the percentage of viable cells in all the other groups decreases. When compared to the control, PB significantly increased cell vitality up to over three times that of the cell after 48 h (280.32%), but AH and PBAH significantly decreased cell viability, notably at 48 h (18.85%; 45.20%) and 72 h (13.76%; -4.36%). BR is not cytotoxic to HPdLF at any point in time (79.56%, 82.19%, and 90.75%) but PBBR is cytotoxic to cells at 24 and 48 h (66.15%; 65.34%).

In this study, only PBAH demonstrated a statistically significant improvement in cell viability (45.2%) after 48 h compared to AH (18.85%) in the presence of PB ($p < 0.05$). Additionally, the PCV of PB increased from 24 to 48 h in each group's trend throughout time intervals before declining slightly at 72 h. From 24 to 72 h, PCV significantly decreases in AH and PBAH ($p < 0.05$). However, BR and PBBR show no noticeable rise in PCV over time and stay steady, and PBBR has a lower PCV than BR, although this difference is not significant ($p > 0.05$).

Cytotoxicity is an important aspect of the root canal sealer, as it might leach out of the root canal system after contact with moisture. Therefore, both fresh and set sealers were assessed in the *in vitro* cytotoxicity test over the period of 72 h via the MTT assay on HPdLF. Generally, the AH and PBAH groups were both significantly more cytotoxic ($p< 0.05$) than BR and PBBR. This finding is consistent with earlier research **(**Eldeniz et al. 2016; Jung et al. 2019; Taraslia et al. 2018; Wuersching et al. 2022**)** that assessed the cytotoxicity of BR and AH on HPdLF. Besides, the outcome of the current study is consistent with findings from other studies in which AH is also cytotoxic to primary human osteoblasts (Jung et al. 2019; Wuersching et al. 2022) as well as immortalised human gingival fibroblasts (Poggio et al. 2017), male

Caucasian mesenchymal stem cells (Wuersching et al. 2022), and human mesenchymal stem cells. The current findings can be compared with those of recent research (Poggio et al. 2017) in which AH showed cytotoxicity, with PCV dropping from 92.95% to 42.47% and then 24.54% at 24, 48, and 72 h. This can be explained by the fact that AH includes cytotoxic epoxy resin. According to Schweikl, Schmalz and Federlin (1998), epoxy resin has been described as a mutagen that has the potential to disrupt cellular DNA.

According to most studies, AH is cytotoxic when it is fresh but not after it has been set (Eldeniz et al. 2007; Silva et al. 2016; Zhou et al. 2015). Yet in this study, AH exhibits cytotoxicity at 48 and 72 h. This could be simply because the toxicity increases with the length of time that HPdLF is induced. Hence, it can be said that AH is cytotoxic in a time-dependent way, which is consistent with the findings of the study by Jung et al. (2019). In research by Eldeniz et al. (2007), aged AH was let to set for 7 days prior to the eluate extraction, whereas fresh AH was mixed and left to set for three times the manufacturer-recommended setting period. This study claimed that aged AH is less cytotoxic than fresh AH. Therefore, the variance in procedure justifies the difference in the outcome.

EEPB is not harmful to healthy human fibroblast cells, according to a prior study (Valle Jr. et al. 2016). Another study testing the cytotoxicity of *Piper betle*-produced zinc oxide nanoparticles towards Balb 3T3 murine fibroblast cell lines also support the conclusion that PB is not cytotoxic (Rao et al. 2018). In contrast, PB is found cytotoxic toward adherent human cervical cancer cell line HeLa (HPV18 positive) cells (Karak et al. 2018) and colon cancer cells (Ng et al. 2014). This does not necessarily imply that PB is completely toxic, but it can suggest possible anticancer properties (Del Socorro, Bendoy & Dacayana 2014). It has been demonstrated in this current study that the PBAH significantly increases PCV at 48 h compared to AH (45.20%; 18.85%) ($p < 0.05$). This may be because of the bioactivity of PB, as seen in group PB, where PCV and cell proliferation are positively impacted.

At 72 h, there is a modest decline in PCV in PB, which may indicate that HPdLF has entered its death phase. When HPdLF is not exposed to any test material, the death phase in EEPB occurs earlier than in the control group. After 72 h, when there is cell confluence in the PB group as the number of cells increases, cell death can be caused by a buildup of toxic chemicals or nutritional starvation. The requirement for resources rises when cell density rises and crosses a particular threshold, which causes nutritional shortage and, in turn, reduced cell-cell communication and mitochondrial damage (Sakagami et al. 2009). This may also account for the negative PCV of PBAH after 72 h, -4.36%.

Due to its capacity to positively impact cell metabolism, BR is not only biocompatible but also bioactive, as concluded by Jung et al. (2019). A considerable increase in cell proliferation, spreading, and attachment was also seen in HPdLF in the presence of BR (Collado-González et al. 2017). The current results of BR are consistent with earlier findings that HPdLF is not cytotoxic as PCV is greater than 70% at all time intervals. We also notice a consistent rising trend in PCV over time. This study's conclusion that BR is less cytotoxic than AH is consistent with other studies **(**Eldeniz et al. 2016; Jung et al. 2018; Poggio et al. 2017; Taraslia et al. 2018; Wuersching et al. 2022**)**. Regrettably, PBBR failed to demonstrate enhanced biocompatibility. When compared to BR alone, PBBR showed slightly reduced PCV, but the difference was not statistically significant ($P > 0.05$). At 24 and 48 h, PBBR is regarded as cytotoxic. This may be a result of the potential consequences of the decreased calcium ion release when PB was added to BR. Consequently, BR's cytocompatibility was subsequently decreased, which in turn decreased cell proliferation. According to previous research, HPdLF and mesenchymal stem cells may begin to mineralize when calcium ions penetrate into the periapical tissue (Jung et al. 2019).

ANTIBACTERIAL

The antibacterial properties of the PB extract combined with sealers in comparison with sealers alone against *E. faecalis* by modified DCT are presented in Figure 4. *E. faecalis* survival is inversely correlated with the antibacterial efficacy of tested materials. Except for PBBR at 7 days, all groups had significant antibacterial effects ($p < 0.05$) when compared to the negative control. *E. faecalis* survival was always zero in both AH and PBAH.

As the setting time increases, at fresh, 24 h, and 7 days, the antibacterial effects of BR (75.67%; 92.83%; 96.6%) and PBBR (95.62%; 96.33%; 99.56%) steadily diminish. Additionally, a larger percentage of *E. faecalis* survived on the agar plate for PBBR compared to BR, indicating less potent antibacterial activity ($p < 0.05$).

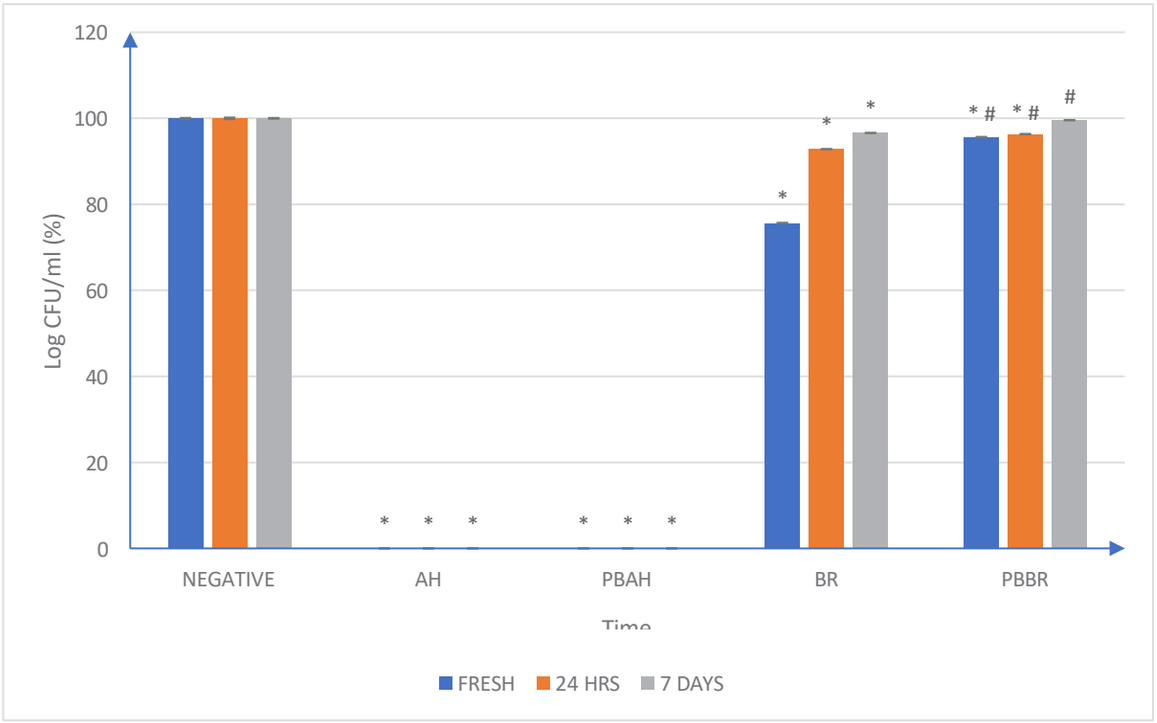

FIGURE 4. Survival of *E. faecalis* strain after direct contact with fresh, one-day-set, and 7-day-set sealer

* : Statistically significant differences compared to the negative group at different time interval ($p < 0.05$)

: Statistically significant differences observed in comparison among commercial group with experimental group ($p < 0.05$)

Since no *E. faecalis* colonies were found using the MDCT technique at any time intervals (fresh, 1 day, or 7 days), AH and PBAH demonstrated 100% eradication of *E. faecalis* in this study. This finding was supported by research that, using a related technique, found full eradication of *E. faecalis* from 1 h to 168 h earlier (Candeiro et al. 2016). But according to a few other research, AH only had an antibacterial impact when it was in its fresh condition (Kayaoglu et al. 2005; Pizzo et al. 2006; Zhang et al. 2009). This is attributed to the release of formaldehyde during the polymerization process of AH when it was in its fresh state (Leonardo et al. 1999).

Additionally, according to a prior study (Candeiro et al. 2016), AH and PBAH have shown superior antibacterial effects than BR and PBBR. In contrast to the findings of a study using the bioceramic endodontic sealer Endosequence BC Sealer (Brasseler, USA), where its antibacterial properties were demonstrated from 24 to 168 h in the Direct Contact Test (DCT) (Candeiro et al. 2016), the PBBR in the current study did not exhibit antibacterial properties at day 7 (99.56% survival of *E. faecalis*). This discrepancy can be caused by the different materials, techniques, or brands employed in the two studies. Instead of using DCT in this investigation, MDCT was chosen because it allows for the evaluation of microorganisms under biofilm settings, which accurately reflect root canal conditions. However, there is also another study found that BR dramatically decreased its antibacterial properties on day 7 (Alsubait et al. 2019), suggesting that this may have been the cause of the variation in ion leaching from the sealer. Additionally, research by Mak et al. (2022) showed that iRootSP, a tricalcium silicate material, had only moderate antibacterial activity when measured by MDCT. High pH values are necessary for bioceramic sealers to have an antibacterial effect. For the calcium and hydroxide ions to create calcium hydroxide, moisture in the root canal is necessary (Camilleri 2011). Therefore, it is hypothesized

that the inadequate antibacterial activity of BR in this study was likely caused by a lack of moisture throughout the material's incubation till day 7.

Because of BR's time-dependent toxic effect, the antibacterial activity of BR and PBBR gradually decreased in this investigation were observed (Alsubait et al. 2018). According to Al-Haddad and Che Ab Aziz (2016), BR's antibacterial actions are dependent on its capacity to produce hydroxyl ions and raise the pH of its immediate surroundings. The acidity of PB, whose pH of PB leaves was observed in a recent study to be 5.98 (Rafi 2021), may be the cause of the current PBBR's lower antibacterial action. According to Valentão et al. (2010), the high amount of organic acids is likely to blame for the low pH of PB. According to published research, piper leaves extracted with hot water had a pH of 4 (Sari & Isadiartuti 2006).

Conclusions

Within the constraints of the study, it can be concluded that PBAH had much less cytotoxicity on HPdLF at 48 h compared to AH. At 24 and 48 h, HPdLF was cytotoxic to PBBR. Both AH and PBAH demonstrated antibacterial activity against *E. faecalis*, followed by BR and PBBR in terms of antibacterial properties. Therefore, BR incorporation with PB is not recommended. However, there is a possibility that PB added to AH producing an improved root canal sealer. PBAH is a potential enhanced root canal sealer and may be further studied to fully elicit its profound properties.

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