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ORIGINAL ARTICLE

Cytotoxicity evaluation of combined *piper betle*-bioactive glass on dental pulp stem cells

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ABSTRACT

Recent advances in dentistry emphasize the use of synthetic and natural bioactive materials for regenerating the dentine-pulp complex. This study assesses the cytotoxic effects of *Piper betle*-conditioned media, alone and combined with bioactive glass 45S5 Bioglass. Three test groups were used: *Piper betle*-only, Bioactive glass-only, and the combined *Piper betle*-Bioactive glass. Cytotoxicity was measured using the MTT assay on dental pulp stem cells on day one, two, four, seven, and fourteen. Cells exposed to 4.00 mg/ml Bioactive glass-only showed the highest cell viability, while *Piper betle*-only demonstrated the highest viability at a concentration of 31.25 µg/ml. The combined *Piper betle*-Bioactive glass showed the best results at 4 mg/ml and 30 µg/ml. Overall, the combination group had the highest cell viability, followed by the Bioactive glass-only and *Piper betle*-only groups. No significant differences were found between the combination and Bioactive glass-only groups, but significant differences existed between the combination and *Piper betle*-only groups. In conclusion, the combination of *Piper betle* and Bioactive glass enhances dental pulp stem cell proliferation, warranting further investigation into its regenerative potential.

Keywords: herb, natural product, regeneration, regenerative endodontic

INTRODUCTION

The dental materials field has evolved significantly over the past century. Bioactive glass (BG), renowned for its bone-bonding and tissue regeneration properties, has been increasingly explored in combination with natural compounds to augment its biological performance.[1,2] Building on the concept of using BG as a substrate, researchers have explored its potential for incorporating natural herbs and plant extracts. This approach aims to not only enhance the biological performance of BG but also introduce a sustainable, nature-derived biomaterial for various medical and dental applications. Plant extracts have been shown to play a key role in the eco-friendly and safe reduction and stabilisation of metal ion nanoparticle.[3] There is a gap of knowledge in relation to the bioactive potential of *Piper betle* (PB) extract towards dental pulp stem cells (DPSC). To the best of our knowledge, there is no study to date analyzing the effect of PB alone and in combination with BG towards the proliferation of DPSC. Thus, it is important to investigate PB potential in enhancing the bioactivity of BG. The purpose of this study is to evaluate the cytotoxicity of PB alone, BG alone and PB in combination with BG. Both materials have shown promise individually in promoting cellular growth and tissue healing, particularly in dental applications. This study aims to assess whether combining these two bioactive agents can enhance dental pulp stem cell viability, potentially offering a novel therapeutic approach for dental tissue engineering. These combinations offer potential improvements in tissue regeneration, antimicrobial properties, and overall biocompatibility, making them promising candidates for next-generation biomaterials.

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LITERATURE REVIEW

Recent research in dental biomaterials and natural products has increasingly focused on the novel integration of plant-based extracts with engineered biomaterials to achieve a synergistic therapeutic effect. A major advancement in dentistry has been the use of biocompatible, regenerative substances that aid in pulp protection, stimulate dentine bridge formation, and optimize pulp preservation.[4–7] Bioactive glass (BG) is particularly recognized for its biodegradable nature and its ability to release biologically active ions that bond with bone and promote tissue regeneration.[8] The versatility of BG has enabled its application in various dental fields, including restorative materials, mineralizing agents, and coatings for dental implants, as well as in procedures like pulp capping, root canal treatments, and air abrasion.[9,10]

Recent efforts to enhance the biological properties of BG have involved incorporating natural compounds, which has led to its consideration in various biomedical applications.[11] Schuhladen et al.[11] conducted a focused review on the potential of combining phytotherapeutics with BG for applications ranging from antibacterial materials to tissue engineering and anti-cancer therapies. *Piper betle* (PB) stands out as a plant widely grown across Southeast Asia and has been used in Ayurvedic and traditional Chinese medicine, as well as in modern Western practices. The therapeutic potential of PB has been well-researched due to its ease of availability and minimal side effects, positioning it as a viable alternative to conventional treatments.[3,12,13] *Piper betle* extracts exhibit a range of biological activities, including antibacterial, antifungal, anti-inflammatory, anticancer, and antioxidant properties, making it an ideal candidate for further investigation in dental applications.[14,15] It contains various biologically active compounds, the concentrations of which depend on factors such as plant variety, season, and climate. Key phenolic compounds identified in PB include hydroxychavicol, chavibetol, eugenol, and chavicol.[12] Of these, hydroxychavicol is considered the most significant, owing to its potent antioxidant, antimicrobial, and anti-inflammatory effects.[16]

In the fields of regenerative medicine and stem cell-based tissue engineering, adult stem cells, particularly dental pulp stem cells (DPSC), have demonstrated significant promise due to their self-renewal capacity, rapid proliferation, and pluripotent potential.[17] Research on incorporating stem cell and herb such as PB with bioactive glass in dentistry is still an emerging field, but there have been promising studies exploring the potential of these components for regenerative purposes.[3] Not many products are available in the market combining bioactive glass with plant extract. Furthermore, no study has been reported regarding the combination of BG with PB extracts. This study addresses this gap by investigating the potential of PB extracts and BG, both alone and in combination, in promoting DPSC viability. The findings from this preliminary study could provide valuable insights into the potential of PB and BG as bioactive agents in next-generation dental biomaterials aimed at improving tissue regeneration and biocompatibility.

METHOD

The fresh betel leaves were collected from Kota Bharu, Kelantan, Malaysia and was taxonomically classified by the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang (voucher number: Herbarium 11877). The plant was confirmed as *Piper betle* Linn species from the *Piperaceae* family. A total of three experimental groups were evaluated for cytotoxicity towards DPCS: BG-only, PB-only and BGPB (BG combined with PB). DPSC were purchased from LONZA, USA (PT-5025). Cells at passage six, seven and eight were used in this study. The PB ethanolic extracts were prepared by adopting previously published methods using a modified maceration technique.[18,19] The sol-gel 45S5 BG were synthesized following to the previous study, by mixing deionized water, 2N nitric acid, tetraethoxysilane, triethylphosphate, sodium nitrate and calcium nitrate.[20] The sol-gel BG powder obtained for this study had a particle size of less than 38 µm. The methodologies for preparing PB-only and BG-only were adapted from previously established protocols, ensuring consistency and reproducibility in the experimental setup.[20,21] The BGPB group was prepared after obtaining the results of the MTT (3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay towards DPSC of BG-only and PB-only. The concentration of BG-only showing the highest DPSC viability was combined with a range of suitable concentrations of PB-only which exhibits an increase in DPSC cell viability.

Prior to the experiment, BG-only, PB-only and BGPB were prepared at various concentrations through serial dilution. For the assay, cells were seeded at a density of $5x10^3$ cells/cm^2 in 96-well plates based on a previous study.[20] The response of DPSC upon exposure towards BG-only, PB-only and BGPB were evaluated using MTT assay (Invitrogen, UK). Briefly, once the cells reached 90% confluence, they were trypsinized, counted, and seeded in 96-well plates. The DPSC were treated with the BG-only (1, 1.5, 2, 4 and 6 mg/mL), PB-only (7.81, 15.63, 31.25, 62.50, 125.00, 250.00 and 500.00 µg/mL) and BGPB (4 mg/mL of

BG with 0.93, 1.87, 3.75, 7.50, 15.0 and 30 µg/mL of PB) followed by incubation. At designated time points (days 1, 2, 4, 7 and 14), the conditioned medium was removed from the well plates and 10.0 µL of MTT solution was added into each well. The plates were then incubated for four hours in an incubator with 5% CO2 at 37°C. Then MTT solution was discarded and 100.0 µL of dimethyl sulfoxide was added into each well. The absorbance was measured using the ELISA microplate reader (Sunrise, TECAN, Austria) at wavelength 570 nm with reference wavelength 620 nm. Data were analyzed using SPPS version 27 with repeated-measure ANOVA and Bonferroni post-hoc test. The results represent the mean values ± standard deviations (Mean±SD) of three independent experiments with four replicates for each experimental group. Non-treated DPSC were used as control comparing it with DPSC exposed to all conditioned medium at each time interval points and differences were considered significant when p-values were less than 0.05 ($p < 0.05$).

RESULTS

Cytotoxicity of DPSC exposed to experimental groups were determined using MTT colorimetric assay. In Figure 1 shows the viability of DPSC upon exposure to BG-only at various time intervals. This is important to ensure that specific dose of BG powder to cell culture medium ratio is suitable for future analysis upon mixing of BG powder with PB extracts. Dental pulp stem cells proliferation increased progressively from day 1 to day 7 in a dose-dependent manner, demonstrating the potential bioactivity of BG. However, cell viability at all concentrations of BG-only group decreased and were significantly lower compared to the control group at day 14. Cell viability was reported to be the highest when cultured in 4.00 mg/mL of BG-only group on day 1 to 7. On day 4 and 7, cell viability in 4.00 mg/mL of BG-only group was significantly higher compared to control group. Dental pulp stem cells exposed to higher BG concentration (6.00 mg/mL) demonstrated significantly lower proliferation ($p < 0.05$) as incubation time increased. Thus, 4 mg/mL BG powder to liquid ratio combined with PB extracts were selected and further studied. The DPSC responses towards PB extracts were assessed using MTT assay are shown in Figure 2. This is important to ensure which dose is suitable for DPSC prior to combining the BG with PB extracts. Dental pulp stem cells were exposed to PB extracts with concentration ranging from 7.81, 15.63, 31.25, 62.50, 125.00, 250.00 to 500.00 µg/mL. The percentage cell viability of DPSC exposed to PB-only group show an increasing trend from day 1 to 2 but decreased from day 4 to 7. Group PB-only at a concentration of 500 µg/mL had the lowest cell viability on day 1, while from day 2 to 14, the lowest cell viability was reported at concentration of 250 µg/mL. A significantly lower cell viability was reported for PB-only group at 250 µg/mL and 500 µg/mL compared to control group throughout the incubation period. The PB-only group at concentrations 62.50 µg/mL and 125.00 µg/mL also reported significantly lower percentage cell viability than the control group. Throughout the observation period, PB-only group at 31.25 µg/mL showed the highest cell viability, with significant difference to control group on day 2. Hence, this concentration of PB extract was selected for further analysis to be combined with BG powder.

Group BGPB was prepared with 4 mg/mL of BG combined with 30 µg/mL of PB, which was subsequently serially diluted to provide a range of suitable concentrations of BGPB. Figure 3 showed the proliferation trend of DPSC exposed to BGPB throughout incubation period. Cell viability DPSC exposed to BGPB group displayed an increasing trend in proliferation from day 1 to 4 but decreased from day 7 to 14. All concentrations of BGPB group reported a significantly lower percentage of cell viability compared to control group on day 1. At day 4, BGPB group at 4 mg/30 µg had significantly higher cell viability compared to control group. Dental pulp stem cells cultured in 4 mg/30 µg also displayed higher viability compared to control group on day 7. Day 14 exhibited significantly lower cell viability compared to control group from concentration 4 mg/3.75 ug to 4 mg/30.00 ug. The decrease in DPSC viability might be because cells have reached the confluence and may have entered a differentiation phase.

The results of the MTT assay, when compared between groups, as shown in **Table 1**. The BGPB group exhibited the highest cell viability followed by BG-only and PB-only groups. No significant difference was found when comparing BGPB with BG-only groups. However, significant results were found when BGPB was compared to the PB-only groups.

Dose-response graphs and inhibitory concentrations at 50% (IC_{50}) were calculated and plotted. The results showed that the inhibitory effects of BG-only, PB-only and BGPB groups were greatly affected by their concentration. The concentration that inhibits 50% of DPSC proliferation following 24 hours of exposure to BG-only, PB-only and BGPB groups were indicated in Table 2.

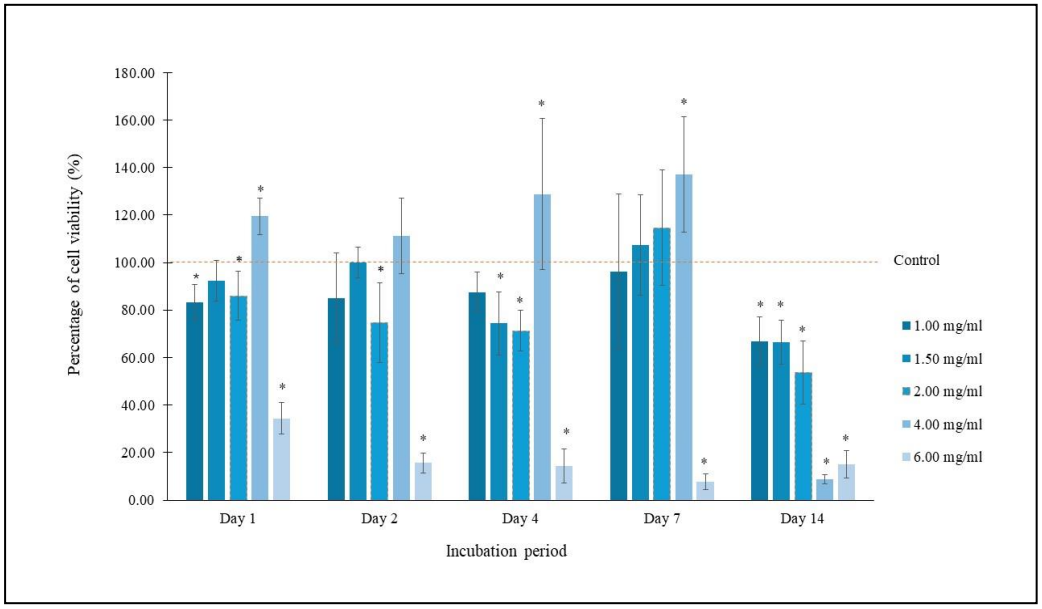

Figure 1. The DPSC cell viability upon exposure to BG-only.
(*denotes significant difference of the marked bar compared to DPSC incubated with control media at the same time point.)

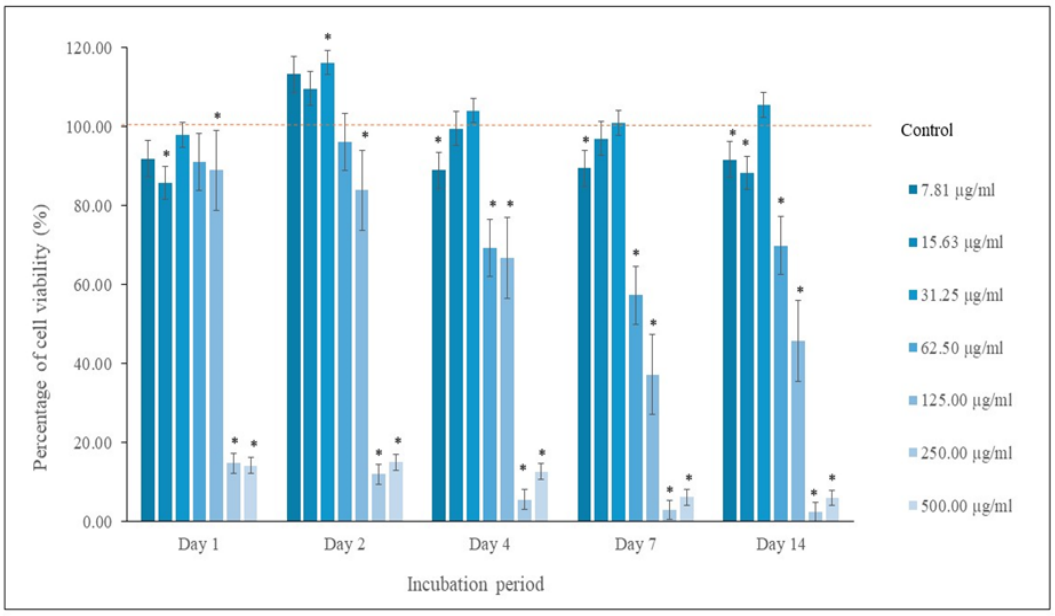

Figure 2. The DPSC cell viability upon exposure to PB-only.
(*denotes significant difference of the marked bar compared to DPSC incubated with control media at the same time point.)

DISCUSSION

The purpose of this *in vitro* experimental study was to investigate the cytotoxicity effects of BG, PB and combination of BG and PB towards DPSC. The incorporation of BG with PB extract holds significant potential for the development of an innovative pulp capping dental material. Such materials, when combined with PB well-known antimicrobial and anti-inflammatory properties, could offer enhanced bioactivity, promoting the migration, proliferation and differentiation of DPSC. Evaluating their bioactivity and ensuring minimal cytotoxicity is crucial, particularly in biologically driven therapies aimed at preserving pulp vitality. Beyond pulp capping, this combination could have broader applications in regenerative endodontics, including regenerative periapical surgery, bone regeneration, and perforation repair in non-stress-bearing areas. The potential for BG and PB to synergistically promote tissue regeneration while minimizing adverse effects could be transformative for modern dental practices.

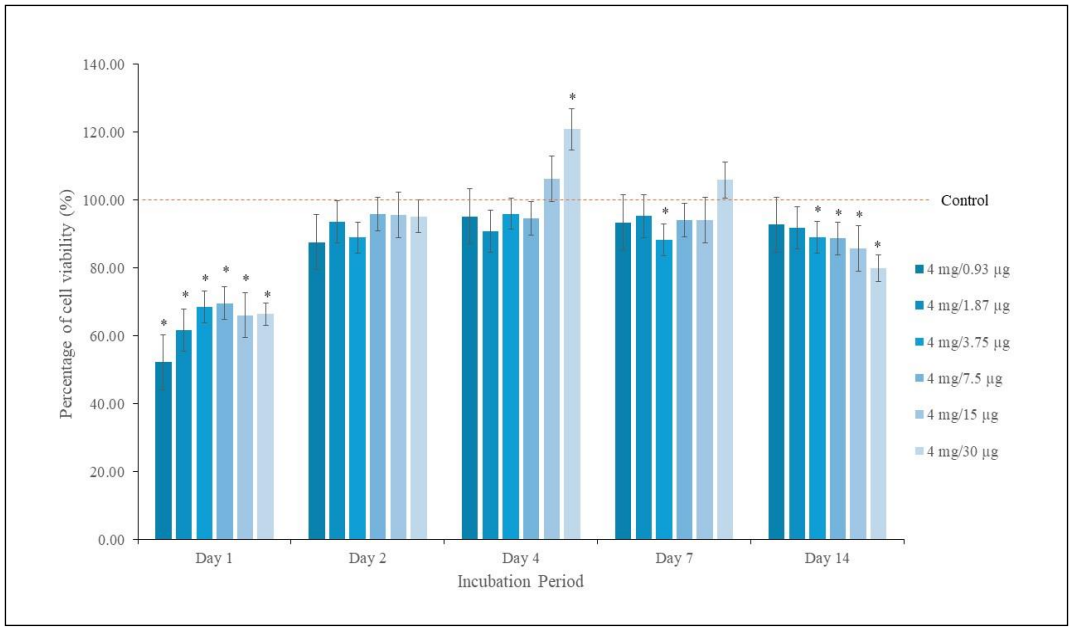

Figure 3. The DPSC cell viability upon exposure to BGPB.
(*denotes significant difference of the marked bar compared to DPSC incubated with control media at the same time point.)

Table 1. Comparison between BG-only, PB-only and BGPB

Pairwise comparison	Mean difference (SD)	*P*-value	95% Confidence Interval	
			Lower Bound	Upper Bound
BG vs PB	9.01 (7.62)	0.72	-9.623	27.639
BG vs BGPB	-13.39 (7.85)	0.27	-32.580	5.805
PB vs BG	-9.01 (7.62)	0.72	-27.639	9.623
PB vs BGPB	-22.40 (7.30)	0.01*	-40.250	-4.542
BGPB vs BG	13.39 (7.85)	0.27	-5.805	32.580
BGPB vs PB	22.40 (7.30)	0.01*	4.542	40.250

[a] Repeated Measure ANOVA test with Bonferroni post-hoc comparisons test.
* Significance level was set at p <0.05

Table 2. IC_{50} values of BG-only, PB-only and BGPB at 24 hours

Group	IC_{50} Value
BG-only	2.69 mg/mL
PB-only	4.77 µg/mL
BGPB	4 mg/3.42 µg

Ethanol has been used as the solvent in this study as it has been found to be more effective in extraction of phenolic compounds. Recent study reported that higher amount of phenolics were extracted by using ethanol as solvent compared to methanol, ethyl acetate and hexane.[22] The primary phytochemicals in PB are highly polar, making them soluble in a highly polar solvent.[23] Ethanol proved to be the most effective solvent for extracting phenolic compounds like chavicol, hydroxychavicol, chavibetol, chavibetol acetate, and eugenol.[16]

In general, all tested group has similar trend of gradual increase in cell proliferation from day 1 to 7. The MTT colorimetric assay revealed that lower BG powder-to-liquid ratios (1 to 4 mg/mL) significantly enhanced DPSC viability. However, BG at a concentration of 6 mg/mL showed toxic effects on DPSC compared to lower BG doses. These findings align with previous studies, which also observed that lower BG concentrations promote higher cell viability, whereas higher concentrations result in reduced viability.[21,24,25] The observed effects can be attributed to BG's dissolution, releasing biologically active ions including calcium, phosphorus, silicon, sodium, and strontium, which are known to play a key role in cellular proliferation and tissue regeneration.[26] The increased levels of these ions within the first 24 hours contribute to their ability to induce cell proliferation. Specifically, the elevated release of silicon ions from BG may play a crucial role.[25] However, higher concentrations of these ions can affect gene expression by up-regulating certain pathways, which ultimately leads to reduced cell proliferation.[27] Meanwhile, Noor et al.[20] found that BG at 4 mg/mL lowered DPSC viability compared to 1 mg/mL and 2 mg/mL. This may be due to higher silicon, calcium, and phosphorus ion levels in the conditioned media, which can enhance metabolic activity at higher concentrations.[28] Thus, cell behavior may be directly or indirectly influenced by changes in ionic levels.

Cells treated with PB-conditioned media exhibited higher proliferation compared to BGPB group at early incubation days. This finding is aligned with previous study that use plant of the similar species, Piper sarmentosum. It was reported a significant increase of viable human peripheral blood stem cells ($p < 0.05$) starting from day 1 to day 14, with approximately 34-fold increase on day 14 compared to day 0 after treated with *Piper sarmentosum* ethanolic extract.[29] *Piper betle*-only group displayed potential for promoting DPSC proliferation at an effective dosage of 31.25 µg/mL, with cytotoxicity noted at concentrations of 250.00 µg/mL and 500.00 µg/mL.

The initial reduction in viability on day 1 in BGPB group may be due to the cells needing time to adapt to the new environment created by the combination of culture media and the extract. Notably, BGPB group at 4.00 mg/30.00 µg resulted in higher cell proliferation compared to the control on day 4 and 7, suggesting an optimal dose of BGPB for promoting cell growth. At day 14, the metabolic activity of DPSC was lower than day 7. This may be due to the higher number of cells present within the 96-well plate, which resulted in cell death due to the lack of nutrients. The lower DPSC metabolic activity may also have occurred when they became too confluent, where they might have experienced contact inhibition. Although cell proliferation of PB-only and BGPB groups decreased on day 14, the percentage of DPSC viability was above 70%. This indicated that PB-only and BGPB groups, at these concentrations were not cytotoxic and further research is warranted.

It was shown that BGPB group can promote the viability of cell, which suggests that mixed BG powder and PB extract may be effective for increasing proliferation of DPSC. The concentrations of the BGPB group used in this study effectively promoted DPSC viability without disrupting cell metabolic activity, demonstrating its potential for enhancing cell growth. However, further clarification is required to support these findings. Future research should explore the interaction between BG and PB leaf extracts, particularly whether they form a complex structure, which could be confirmed through other analysis such as nuclear magnetic resonance spectroscopy. Investigating the chemical interactions between BG, PB, and other active compounds within the PB leaf extract is also essential to better understand the mechanisms behind the improved DPSC response to the BG and PB combination.

CONCLUSION

In conclusion, patients with T2DM who experience ED have shown that ED adversely affects their sexual lives, causing emotional disturbances and relationship problems. However, these men expressed hope for treatment. The assessment and exploration of the impact of ED in men with T2DM should be initiated by healthcare professionals who directly support this population during their consultations.

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