



Original Article

Piper betle Phytochemical Analysis and Cytotoxicity Evaluation of Combined Bioactive Glass – *P. betle* on Dental Pulp Stem Cells

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ABSTRACT

Objectives: Recent advances in endodontics emphasize regenerative strategies, utilizing both synthetic and natural bioactive materials to protect and regenerate the dentine-pulp complex. *Piper betle* (PB), a member of the Piperaceae family, possesses various medicinal properties. This study aims to analyze the phytochemical compounds of PB extract, evaluate the cytotoxicity of PB-conditioned media both individually and in combination with bioactive glass (BG) 45S5 Bioglass®, and identify the chemical compounds present in optimal doses of PB and BG-conditioned media, separately and in combination (bioactive glass-piper betle [BGPB]).

Material and Methods: The PB extract was prepared by ethanol maceration and analyzed using gas chromatography–mass spectrometry (GCMS) analysis to identify bioactive phytochemicals present. Three test groups were utilized (PB, BG, and BGPB-conditioned medium). Each test group's conditioned medium was prepared at various concentrations through serial dilution. Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on dental pulp stem cells (DPSC) on days 1, 2, 4, 7, and 14. The optimal concentration dose of each test group underwent GCMS analysis. Data were analyzed using repeated-measures analysis of variance with Bonferroni *post hoc* tests (Statistical Package for the Social Sciences v27; $P \leq 0.05$).

Results: The GCMS analysis of the PB extract revealed the presence of phenols, esters, ketones, carbohydrates, and nucleotides. DPSCs exposed to 4.00 mg/mL BG-conditioned media showed the highest cell viability, while cells cultured in PB-conditioned media exhibited the highest cell viability at a concentration of 31.25 µg/mL. Hence, these concentrations of PB and BG were selected for combination in further analysis. The BGPB-conditioned media showed the highest percentage of viable cells at a concentration of 4 mg/30 µg. Overall, the BGPB group demonstrated the highest cell viability, followed by the BG and PB groups. No significant difference was observed between the BGPB group and the BG group, but significant differences were found when comparing the BGPB group to the PB group. However, no significant difference in cell viability was detected between the BG and PB groups. Various active compounds, including phenolic compounds, terpenoids, esters, fatty acids, ketones, furans, imines, siloxanes, and amides, were detected in BG, PB, and BGPB-conditioned media.

Conclusion: Within the limitations of this study, BGPB has shown potential to enhance the proliferation of DPSC *in vitro* at specific dosages and warrants further investigation for its regenerative properties.

Keywords: Bioglass 45S5®, Dental pulp stem cells, Phytochemicals, *Piper betle*, Proliferation

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INTRODUCTION

Successful dental restorative treatment outcomes require the interfacial adhesion of dental material to surrounding soft or hard tissue adequate to withstand dislodging forces.^[1] Bioactive materials have emerged as a pivotal innovation, capable of interacting biologically with tissues to form robust interfaces. Among these, bioactive glass (BG), particularly the 45S5 Bioglass®, has gained prominence for its capacity to form a hydroxycarbonate apatite layer upon reacting with tissue fluids, facilitating hard and soft tissue regeneration.^[2] This material has been extensively applied in dentistry for enamel and dentine remineralization, pulp capping, and cavity lining.^[3]

Similarly, plant-derived biomaterials have garnered interest for their bioactivity and safety.^[4] *Piper betle* (PB), a plant traditionally used in Southeast Asia for its medicinal properties, exhibits antimicrobial, antioxidant, and wound-healing capabilities.^[5] Research suggests that PB can reduce acid production by *Streptococcus mutans*^[6] and enhance fibroblast proliferation,^[7] making it a promising candidate for dental applications. While PB has been incorporated into products such as toothpaste^[8] and mouth rinses^[9] for its anticaries properties, its potential in dental tissue regeneration remains underexplored.

Many novel dental restorative and regenerative materials have been tested for their effects on dental pulp stem cells (DPSC) proliferation, viability, and differentiation. DPSCs are increasingly important in regenerative medicine and dentistry. However, there is a gap in knowledge regarding the bioactive potential of PB extract toward dental stem cells. Therefore, it is important to evaluate the cytotoxicity of PB extract on dental tissues and cells, as well as its ability to enhance the bioactive potential of synthetic biomaterials like BG. The present study aims to evaluate PB's potential in enhancing the bioactivity of BG. Notably, no previous research has examined the effects of PB alone or in combination with BG on the viability of DPSCs. This preliminary study focuses on the phytochemical screening of PB extract and the cytotoxicity of BG combined with PB extract on DPSC.

MATERIAL AND METHODS

Study area

This study was conducted in the Craniofacial Science Laboratory, School of Dental Sciences, Universiti Sains Malaysia Health Campus, Kubang Kerian, Kelantan, and Advanced Medical and Dental Institute, Universiti Sains Malaysia, Penang.

Study design

This *in vitro* experimental study aims to identify the chemical compounds present in ethanolic PB extract and evaluate the cytotoxicity of PB, BG and their combination on DPSC as

illustrated in Figure 1. This study was exempted from ethical review as the cells used were commercially obtained.

Methods

Phase I: Analysis of phytochemical compounds in PB extract

Preparation of PB ethanolic extract

The taxonomic identity of PB *L.* was determined by taxonomists in the Herbarium Centre, Department of Biology, Universiti Sains Malaysia, Pulau Pinang, Malaysia (Voucher Number: 11877), confirming it as PB Linn, family Piperaceae. PB ethanolic extract was prepared following Nair *et al.*^[10] with modified time settings. The maceration technique was used in this study where ground samples were soaked in a stoppered container with a solvent and allowed to stand at room temperature for 3 days with frequent agitation.^[11] The powdered sample was extracted with 70% ethanol with a ratio of 1:10 (sample: solvent) and placed on a rotary shaker for 72 h. It was then filtered with Whatman filter paper no.1 and centrifuged at 150 rpm for 15 min. The supernatant was collected and the solvent evaporated using a rotary evaporator (Rotavapor R-210, Buchi) at 40°C, freeze-dried (Scanvac CoolSafe Free Dryer), and stored in a -20°C freezer until use.

Gas chromatography-mass spectrometry (GCMS) analysis of crude PB extract

Crude ethanol extract of PB was analyzed for chemical compounds following Samsurrijal *et al.*^[12] An Agilent 8890/5977C GC/MSD system coupled with an Agilent 8697 headspace sampler and HP-5 MS capillary column (30 m × 0.25 mm × 0.25 μm) was used. Helium served as the carrier gas (1 mL/min), with 1 μL injection volume. Oven temperature was programmed from 40°C to 250°C (2.5°C/min). Injection was in split mode (1:10) with port temperature at 220°C. Mass spectrometry used 71 eV ionization. The total ion chromatograms (TICs) and mass spectra were recorded using MassHunter Unknowns Analysis software and compared against the National Institute of Standards and Technology 20 database.

Phase II: To determine the cytotoxicity effects of BG, PB, and β-glycerophosphate (BGPB) on DPSC using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Preparation of sol-gel BG 45S5 Bioglass®

The sol-gel BG 45S5 Bioglass® obtained was synthesized according to a previous study by Noor *et al.*^[13] by mixing the reagents of deionized water, 2N (normality) nitric acid,

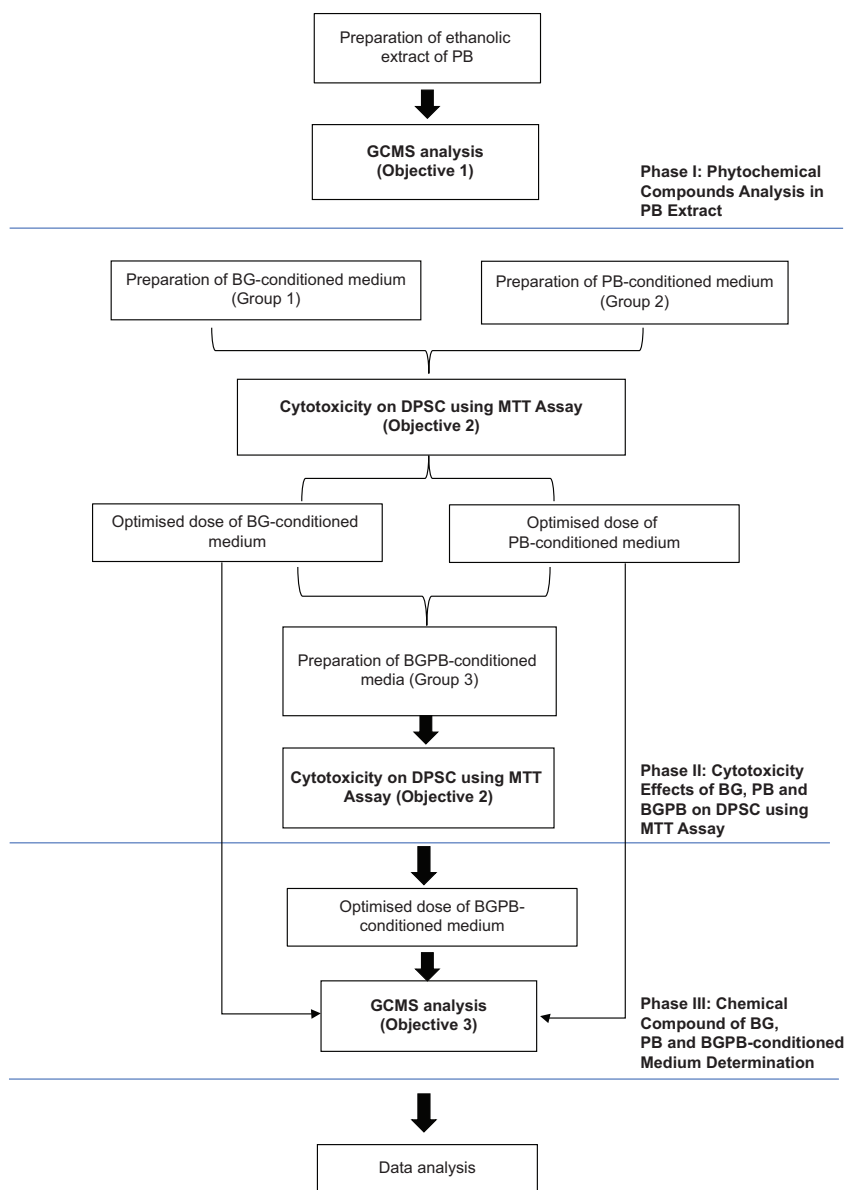


Figure 1: Flowchart of study.

tetraethoxysilane, triethylphosphate, sodium nitrate, and calcium nitrate. The BG 45S5 Bioglass® composition in weight percentages (wt%) and mole percentages (mol%) is shown in Table 1.

The mixture was poured into a sealed container, oven dried for 2 days at 60°C, followed by 110°C, then sintered at 600°C for 1 h to produce sol-gel frits. The frits were milled using a planetary micro mill (Pulverisette, Germany) to obtain a fine powder and mixed with PB extracts.^[12]

Preparation of BG- and PB-conditioned media

BG-conditioned media were prepared as described by Noor et al.^[13] The sol-gel BG powder (<38 µm) was mixed with

Table 1: The bioactive glass 45S5 Bioglass® composition in weight percentages (wt%) and mole percentages (mol%).

Composition	Weight percentages (wt%)	Mole percentages (mol%)
SiO ₂	45.00	46.10
Na ₂ O	24.50	24.40
CaO	24.50	26.90
P ₂ O ₅	6.00	2.50

Dulbecco’s Modified Eagle Media (DMEM; Gibco, USA) to obtain concentrations of 1, 2, 4, and 6 mg/mL by serial dilution. PB-conditioned media were prepared similarly, with freeze-dried PB extract dissolved in DMEM to obtain

concentrations of 7.81, 15.63, 31.25, 62.50, 125.00, 250.00, and 500.00 µg/mL by serial dilution.^[12,14] Both media were incubated at 37°C with 5% CO₂ for 4 h in 50 mL sterile polypropylene tubes sealed with paraffin film, followed by shaking at 100 rpm for 4 h. The mixtures were then filtered through a 0.22 µm syringe filter, supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% antibiotic/antimycotic (A/A; Gibco, USA), and incubated overnight at 37°C with 5% CO₂ before use.

Preparation of BGPB-conditioned media

The BGPB-conditioned media were prepared after obtaining the results of the MTT assay of BG and PB-conditioned media toward DPSC. The concentration of BG-conditioned media showing the highest DPSC viability was combined with a range of suitable concentrations of PB-conditioned media which exhibits an increase in DPSC cell viability.

The BG powder and PB extracts were weighed using an electronic balance and subsequently mixed with the DMEM cell culture media, followed by incubation in an incubator shaker for 4 h at 37°C and 5% CO₂. Then, the BGPB-conditioned media were filtered using a 0.22 µm syringe filter. The conditioned media were supplemented with 10% FBS and 1% A/A and incubated in a CO₂ incubator overnight before use.

Evaluation of cell viability (%) of BG, PB, and BGPB

Cell viability assessment of BG and PB extract was conducted individually and in combination toward DPSC using the MTT assay. MTT assay was conducted for BG- and PB-conditioned media. A negative control group consisting of cells in complete growth media was also included and wells with no cells were used as blanks. The absorbance was measured using the enzyme-linked immunosorbent assay microplate reader (Sunrise, TECAN, Austria) at a wavelength of 570 nm with reference wavelength 620 nm. The percentage cell viability was calculated using the following formula^[15].

$$\frac{\text{OD obtained for sample}}{\text{OD obtained for control}} \times 100 \\ = \text{Percentage viability of the cells}$$

The concentration ratios of BGPB-conditioned media were determined based on the results obtained from the MTT assay conducted on BG- and PB-conditioned media. The concentration of BG with optimal DPSC viability was combined with a range of concentrations of PB extract which showed an increase in DPSC viability. MTT assay was then conducted for BGPB-conditioned media following the same methods as described above.

Phase III: To determine the chemical compounds of the optimal dose of BG-, PB- and BGPB-conditioned media

The optimal dose of BG-, PB-, and BGPB-conditioned media with the highest percentage cell viability was subjected to GCMS analysis using the same method used for crude PB extract phytochemical analysis. The Agilent 8890/5977C GC/MSD system, coupled with an Agilent 8697 headspace sampler, was also used.

Data analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 27 (IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp). The results were represented by the mean (standard deviation) for the three test groups. Data from the study were found to be normally distributed. As such, a repeated-measure analysis of variance test was used to determine the association between the variables within each test group and between test groups. When a statistically significant difference was found, further statistical analysis was conducted with the Bonferroni *post hoc* comparison test. The differences were considered significant when $P < 0.05$.

RESULTS

Phase I: Phytochemical compounds analysis in PB extract

PB extract chemical compound determination

The GCMS analysis of PB ethanol extract revealed 200 phytochemicals. The report of the TIC obtained provided details of retention time, name of compounds, molecular formula, and peak area percentage. Ten compounds with the highest percentage peak area are listed in Table 2. The major compounds detected were hydroxychavicol (70.37%), 3-allyl-6-methoxyphenol/chavibetol (17.78%), ethyl beta-d-ribose (1.77%), 2-hydroperoxytetrahydrofuran (0.59%), and trimethylsilyl derivative dihydroxyacetone (0.55%).

Phase II: Cytotoxicity effects of BG, PB, and BGPB on DPSC using MTT assay

The proliferative effects of conditioned media on DPSCs were assessed using the MTT assay, with optical density values indicating cell viability. The findings are reported in accordance with guidelines of the International Organization for Standardization (ISO), which classify cells with a viability level of <70% as cytotoxic (ISO, 2009).

Cytotoxicity of BG-conditioned media on DPSC

The mean percentage cell viability of BG-conditioned media was compared to control media [Table 3]. The

Table 2: Chemical compounds detected in the ethanolic extract of PB.

No.	RT (min)	Compound PB	Group	Peak area (%)
1.	38.0514	Hydroxychavicol	Phenol	70.37
2.	32.7246	3-Allyl-6-methoxyphenol/Chavibetol	Phenol	17.78
3.	44.7440	Ethyl beta-d-ribose	Nucleoside	1.77
4.	44.7125	2-Hydroperoxytetrahydrofuran, TMS derivative	Ester	0.59
5.	8.0052	Dihydroxyacetone	Ketone	0.55
6.	45.6728	Sucrose	Carbohydrate	0.48
7.	55.9596	Benzoic acid, 2-methoxy-6-methyl-, ethyl ester	Phenol	0.45
8.	32.2134	Triacetin	Ester	0.38
9.	39.5893	5-Allyl-2-hydroxyphenyl acetate	Ester	0.32
10.	55.9579	Butan-2-one, 4-(3-hydroxy-2-methoxyphenyl)-	Ketone	0.31

PB: *Piper betle*, RT: Retention time, TMS: Trimethylsilyl

proliferation trend of DPSC exposed to BG-conditioned media throughout the incubation period [Figure 2]. DPSCs displayed increasing proliferation from days 1 to 7 in a dose-dependent manner. However, cell viability at all concentrations of BG-conditioned media decreased and was significantly lower compared to the control group at day 14 ($P < 0.05$). Cell viability was reported to be the highest when cultured in 4.00 mg/mL of BG-conditioned media from days 1 to 7. On days 4 and 7, cell viability in 4.00 mg/mL of BG-conditioned media was significantly higher compared to the control ($P < 0.05$). BG-conditioned media at 6.00 mg/mL were found to be cytotoxic to DPSCs, as it reported significantly lower cell viability compared to control throughout the incubation period ($P < 0.05$). According to these results, the concentration of BG at 4 mg/mL was chosen to be combined with PB extract for further study.

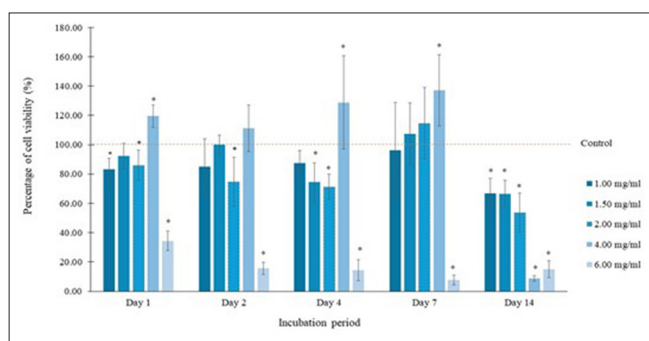


Figure 2: The dental pulp stem cell (DPSC) viability upon exposure to bioactive glass-conditioned media. *denotes a significant difference of the marked bar compared to DPSC incubated with control media at the same time point.

Cytotoxicity of PB-conditioned media on DPSC

The mean percentage of cell viability in PB-conditioned media compared to control media is shown in Table 4. The

proliferation trend of DPSCs exposed to PB-conditioned media over the incubation period is illustrated in Figure 3. The percentage cell viability of DPSCs exposed to PB-conditioned medium showed an increasing trend from day 1 to 2 but decreased from day 4 to 7. PB-conditioned media at a concentration of 500 μ g/mL had the lowest cell viability on day 1, while from days 2 to 14, the lowest cell viability was reported at a concentration of 250 μ g/mL. Significantly lower cell viability was reported for PB-conditioned media at 250 μ g/mL and 500 μ g/mL compared to control throughout the incubation period ($P < 0.05$). PB-conditioned media at concentrations of 62.50 μ g/mL and 125.00 μ g/mL also reported significantly lower percentage cell viability than the control ($P < 0.05$). Throughout the observation period, PB-conditioned media at 31.25 μ g/mL showed the highest cell viability, with a significant difference to control on day 2 ($P < 0.05$). Hence, this concentration of PB extract was to be combined with BG powder for further analysis.

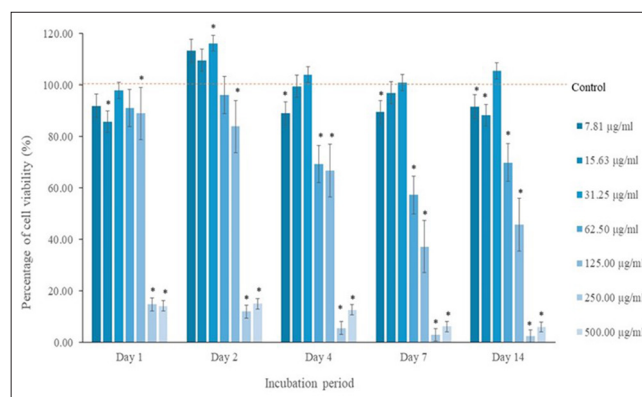


Figure 3: The dental pulp stem cell (DPSC) viability upon exposure to *Piper betle*-conditioned media *denotes a significant difference of the marked bar compared to DPSC incubated with control media at the same time point.

Table 3: Percentage of cell viability of DPSC in BG-conditioned media.

Incubation time	Concentration of conditioned media	Cell viability of DPSC (%) Mean (Standard deviation)	P-value ^a
Day 1	Control	100 (0.00)	
	1.00 mg/mL	83.43 (7.23)	0.01*
	1.50 mg/mL	92.35 (8.41)	0.16
	2.00 mg/mL	86.04 (10.20)	0.02*
	4.00 mg/mL	119.49 (7.60)	0.00*
	6.00 mg/mL	34.41 (6.76)	0.00*
Day 2	Control	100 (0.00)	
	1.00 mg/mL	85.10 (18.81)	0.11
	1.50 mg/mL	100.03 (6.32)	0.99
	2.00 mg/mL	74.68 (16.90)	0.01*
	4.00 mg/mL	111.22 (16.03)	0.22
	6.00 mg/mL	15.59 (4.26)	0.000*
Day 4	Control	100 (0.00)	
	1.00 mg/mL	87.62 (8.39)	0.26
	1.50 mg/mL	74.34 (13.17)	0.03*
	2.00 mg/mL	71.30 (8.66)	0.01*
	4.00 mg/mL	128.74 (31.82)	0.02*
	6.00 mg/mL	14.39 (7.26)	0.00*
Day 7	Control	100 (0.00)	
	1.00 mg/mL	96.19 (32.65)	0.80
	1.50 mg/mL	107.47 (21.12)	0.63
	2.00 mg/mL	114.65 (24.32)	0.34
	4.00 mg/mL	137.16 (24.33)	0.00*
	6.00 mg/mL	7.81 (3.30)	0.00*
Day 14	Control	100 (0.00)	
	1.00 mg/mL	66.87 (10.38)	0.00*
	1.50 mg/mL	66.39 (9.18)	0.00*
	2.00 mg/mL	53.69 (13.36)	0.00*
	4.00 mg/mL	8.75 (1.86)	0.00*
	6.00 mg/mL	14.98 (5.79)	0.00*

^aRepeated measure analysis of variance test with Bonferroni *post hoc* comparisons, *Significance level was set at $P < 0.05$. DPSC: Dental pulp stem cell, BG: Bioactive glass

Cytotoxicity of BGPB-conditioned media on DPSC

BGPB-conditioned media were 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-conditioned media. The mean percentage cell viability of BGPB-conditioned media was compared to control media, as displayed in Table 5. Figure 4 shows the proliferation trend of DPSC exposed to BGPB-conditioned media throughout the incubation period. Cell viability DPSCs exposed to BGPB-conditioned media displayed an increasing

Table 4: Percentage cell viability of DPSC in PB-conditioned media.

Incubation time	Concentration of conditioned media	Cell viability of DPSC (%) mean (standard deviation)	P-value ^a
Day 1	Control	100 (0.00)	
	PB 7.81 µg/mL	91.88 (4.82)	0.14
	PB 15.63 µg/mL	85.72 (2.37)	0.01*
	PB 31.25 µg/mL	97.83 (12.90)	0.69
	PB 62.50 µg/mL	90.98 (6.20)	0.10
	PB 125.00 µg/mL	88.89 (11.01)	0.05*
	PB 250.00 µg/mL	14.74 (7.36)	0.00*
	PB 500.00 µg/mL	14.18 (6.64)	0.00*
Day 2	Control	100 (0.00)	
	PB 7.81 µg/mL	113.25 (7.36)	0.06
	PB 15.63 µg/mL	109.59 (13.75)	0.16
	PB 31.25 µg/mL	116.16 (11.84)	0.02*
	PB 62.50 µg/mL	96.05 (12.40)	0.56
	PB 125.00 µg/mL	83.81 (9.11)	0.02*
	PB 250.00 µg/mL	11.97 (8.92)	0.00*
	PB 500.00 µg/mL	15.01 (3.24)	0.00*
Day 4	Control	100 (0.00)	
	PB 7.81 µg/mL	88.92 (3.64)	0.00*
	PB 15.63 µg/mL	99.45 (4.81)	0.87
	PB 31.25 µg/mL	104.03 (3.95)	0.23
	PB 62.50 µg/mL	69.32 (6.91)	0.00*
	PB 125.00 µg/mL	66.73 (5.18)	0.00*
	PB 250.00 µg/mL	5.56 (1.33)	0.00*
	PB 500.00 µg/mL	12.67 (6.61)	0.00*
Day 7	Control	100 (0.00)	
	PB 7.81 µg/mL	89.40 (1.83)	0.00*
	PB 15.63 µg/mL	96.89 (4.20)	0.28
	PB 31.25 µg/mL	100.85 (3.90)	0.77
	PB 62.50 µg/mL	57.26 (7.57)	0.00*
	PB 125.00 µg/mL	37.18 (4.67)	0.00*
	PB 250.00 µg/mL	3.00 (2.51)	0.00*
	PB 500.00 µg/mL	6.15 (2.55)	0.00*
Day 14	Control	100 (0.00)	
	PB 7.81 µg/mL	91.62 (9.09)	0.03*
	PB 15.63 µg/mL	88.26 (8.66)	0.00*
	PB 31.25 µg/mL	105.43 (5.41)	0.15
	PB 62.50 µg/mL	69.85 (3.76)	0.00*
	PB 125.00 µg/mL	45.68 (3.37)	0.00*
	PB 250.00 µg/mL	2.34 (1.39)	0.00*
	PB 500.00 µg/mL	5.96 (1.67)	0.00*

^aRepeated measure analysis of variance test with Bonferroni *post hoc* comparisons test. *Significance level was set at $P < 0.05$. PB: *Piper betle*, DPSC: Dental pulp stem cell

Table 5: Percentage cell viability of DPSC in BGPB-conditioned media.

Incubation time	Concentration of conditioned media	Cell viability of DPSC (%) Mean (Standard deviation)	P-value ^a
Day 1	Control	100.00 (0.00)	
	BGPB 4 mg/0.93 µg	89.57 (2.84)	0.000*
	BGPB 4 mg/1.87 µg	94.45 (3.95)	0.027*
	BGPB 4 mg/3.75 µg	68.40 (3.61)	0.001*
	BGPB 4 mg/7.50 µg	69.59 (3.77)	0.000*
	BGPB 4 mg/15.00 µg	66.08 (4.52)	0.000*
	BGPB 4 mg/30.00 µg	66.35 (2.11)	0.000*
Day 2	Control	100.00 (0.00)	
	BGPB 4 mg/0.93 µg	87.53 (20.28)	0.293
	BGPB 4 mg/1.87 µg	93.60 (14.12)	0.586
	BGPB 4 mg/3.75 µg	88.89 (16.48)	0.348
	BGPB 4 mg/7.50 µg	95.85 (14.95)	0.723
	BGPB 4 mg/15.00 µg	95.63 (19.01)	0.709
	BGPB 4 mg/30.00 µg	95.16 (20.18)	0.680
Day 4	Control	100.00 (0.00)	
	BGPB 4 mg/0.93 µg	95.17 (4.56)	0.431
	BGPB 4 mg/1.87 µg	90.80 (11.10)	0.141
	BGPB 4 mg/3.75 µg	95.96 (7.11)	0.510
	BGPB 4 mg/7.50 µg	94.53 (8.30)	0.374
	BGPB 4 mg/15.00 µg	106.12 (9.52)	0.321
	BGPB 4 mg/30.00 µg	120.83 (12.38)	0.002*
Day 7	Control	100.00 (0.00)	
	BGPB 4 mg/0.93 µg	93.41 (6.10)	0.152
	BGPB 4 mg/1.87 µg	95.24 (8.11)	0.295
	BGPB 4 mg/3.75 µg	88.23 (8.16)	0.015*
	BGPB 4 mg/7.50 µg	94.07 (7.70)	0.195
	BGPB 4 mg/15.00 µg	94.17 (2.06)	0.203
	BGPB 4 mg/30.00 µg	105.87 (6.50)	0.200
Day 14	Control	100.00 (0.00)	
	BGPB 4 mg/0.93 µg	92.77 (4.81)	0.082
	BGPB 4 mg/1.87 µg	91.80 (6.39)	0.050
	BGPB 4 mg/3.75 µg	89.06 (6.04)	0.012*
	BGPB 4 mg/7.50 µg	88.63 (4.56)	0.009*
	BGPB 4 mg/15.00 µg	85.72 (6.68)	0.002*
	BGPB 4 mg/30.00 µg	79.86 (7.52)	0.000*

^aRepeated measure ANOVA test with Bonferroni *post hoc* comparison test, *Significance level was set at $P < 0.05$. DPSC: Dental pulp stem cell, BGPB: Bioactive glass-piper betle

trend in proliferation from days 1 to 4 but decreased from days 7 to 14. All concentrations of BGPB-conditioned media exhibited significantly lower percentage of cell viability

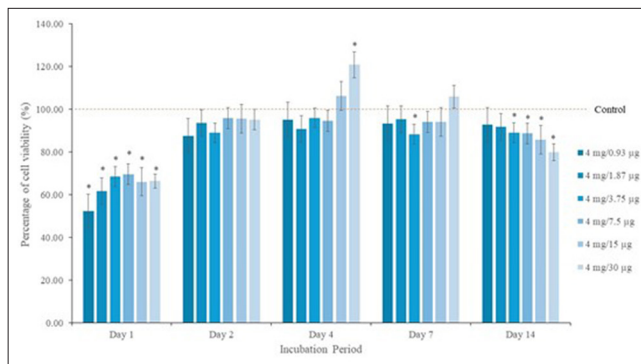


Figure 4: The dental pulp stem cell (DPSC) viability upon exposure to BGPB-conditioned media *denotes a significant difference of the marked bar compared to DPSC incubated with control media at the same time point.

compared to control on day 1 ($P < 0.05$). At day 4, BGPB-conditioned media at 4 mg/30 µg had significantly higher cell viability compared to control ($P < 0.05$). DPSC cultured in 4 mg/30 µg also displayed higher viability compared to the control on day 7. Day 14 exhibited significantly lower cell viability compared to control from concentration 4 mg/3.75 µg to 4 mg/30.00 µg ($P < 0.05$).

Identification of suitable dose in promoting cell proliferation

The results of suitable doses of different conditioned media on the proliferation of DPSCs are presented in Figure 5. DPSCs exposed to 4.00 mg/mL BG-conditioned media demonstrated the highest cell viability on days 1, 4, and 7 compared to PB and BGPB-conditioned media. On days 2 and 14, PB-conditioned media showed the highest cell viability, particularly at concentrations of 31.25 µg/mL and 15.63 µg/mL, respectively. The BGPB-conditioned media exhibited a higher percentage of viable cells than PB-conditioned media on days 4 and 7, with the most effective concentration being BGPB 4 mg/30 µg. On day 14, the lowest cell viability was observed in the 4.00 mg/mL BG-conditioned media, while the 31.25 µg/mL PB-conditioned media reported the highest viability.

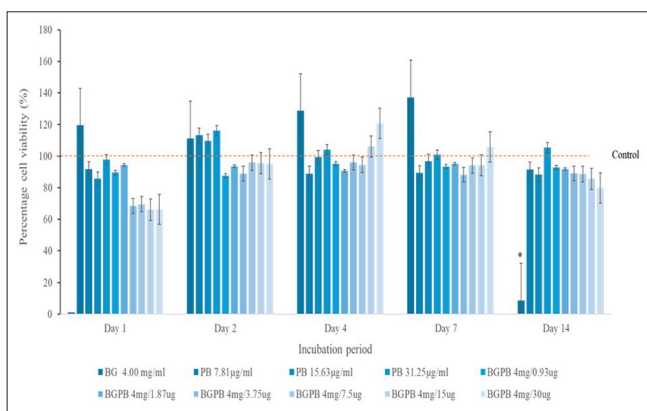
Phase III: Chemical compound of BG, PB, and BGPB-conditioned media determination

Several active compounds have been identified through the GCMS analysis of the test samples. These compounds comprise phenolic compounds, terpenoids, esters, fatty acids, ketones, furans, imines, siloxane, and amide groups. A functional group is an atom or group of atoms within a molecule that has similar chemical properties in different compounds. The classification of functional groups is based on the combination of carbon (C) with other atoms, such

Table 6: The molecular formula of the major chemical compounds detected.

No.	BG-conditioned media		PB-conditioned media		BGPB-conditioned media	
	Chemical compound	Molecular formula	Chemical compound	Molecular formula	Chemical compound	Molecular formula
1.	Oxime-, methoxy-phenyl-	C ₈ H ₉ NO ₂	1-Heptene, 1,3-diphenyl-1-(trimethylsilyloxy)-	C ₂₂ H ₃₀ OSi	2,4-Di-tert-butylphenol	C ₁₄ H ₂₂ O
2.	2-Hydroxy-gamma-butyrolactone	C ₄ H ₆ O ₃	Oxime-, methoxy-phenyl-	C ₈ H ₉ NO ₂	Octadecanoic acid, 2,3-dihydroxypropyl ester	C ₂₁ H ₄₂ O ₄
3.	2,4-Dihydroxy-3-methylbenzaldehyde, 2TMS	C ₁₄ H ₂₄ O ₃ Si ₂	2-Hydroxy-gamma-butyrolactone	C ₄ H ₆ O ₃	2-Hydroxy-gamma-butyrolactone	C ₄ H ₆ O ₃
4.	1,3-Benzenediol, o-(4-methylbenzoyl)-o'-(2-methoxybenzoyl)-	C ₂₂ H ₁₈ O ₅	Terbutaline, N-trifluoroacetyl-O, O, otris (trimethylsilyl) deriv.	C ₂₃ H ₄₂ F ₃ NO ₄ Si ₃	Neophytadiene	C ₂₀ H ₃₈
5.	N-Isobutyl (phenyl) methanesulfonamide	C ₁₁ H ₁₇ NO ₂ S	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	C ₁₆ H ₅₀ O ₇ Si ₈	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂
6.	1-Benzenesulfonyl-1H-pyrrole	C ₁₀ H ₉ NO ₂ S	Cyclononasiloxane, octadecamethyl-	C ₁₆ H ₅₀ O ₇ Si ₈	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	C ₆ H ₈ O ₄
7.	-		3,4-Dihydroxymandelic acid, 4TMS derivative	C ₂₀ H ₄₀ O ₅ Si ₄	5-(Hydroxymethyl) dihydrofuran-2 (3H)-one	C ₅ H ₈ O ₃
8.	-		3,4-Dihydroxyphenylglycol, 4TMS derivative	C ₂₀ H ₄₂ O ₄ Si ₄	Phytol	C ₂₀ H ₄₀ O

BGPB: Bioactive glass-piper betle

**Figure 5:** The dental pulp stem cell viability when exposed to bioactive glass, *Piper betle* and BGPB-conditioned media.

as oxygen (O), hydrogen (H), nitrogen (N), silicon (Si), and halogens such as fluorine (F). In addition, Table 6 displays the molecular formula of the major chemical compounds detected.

DISCUSSION

Chemical components detected in PB extract using GCMS analysis

The present study revealed that the ethanolic extract of PB leaves has several bioactive phytoconstituents with phenolic

compounds being predominant. Hydroxychavicol (70.37%) and chavibetol (17.78%) were the major compounds detected. Hydroxychavicol is consistently reported as the most abundant phytochemical in PB leaves, with a composition ranging from 39.5 % to 69.49%.^[16] Similarly, Gundala *et al.*^[17] reported 26%, while another study detected 69.46% of retention area.^[18] This compound possesses strong biological properties, including antinitrosation, antimutagenic, anticarcinogenic, antioxidant, antimicrobial, and anti-inflammatory.^[15,19] Chavibetol, the second most abundant compound in this study, has also been reported at 2.7% in alcohol extracts^[20] and 25.55% in ethanolic extracts,^[21] respectively. It possesses radioprotective and immunomodulatory, and its derivatives, commonly found in PB essential oils, contribute to the plant's characteristic odor.^[16]

Phenolic compounds such as hydroxychavicol and chavibetol have potent anti-inflammatory and antioxidant properties.^[22] Antioxidants can not only mitigate oxidative stress and improve stem cell survival but also affect the potency and differentiation of these cells.^[23] The difference between the composition of the active compounds of the present study and previous studies may be attributed to factors like the extraction solvent and methods^[15] as well as the variety of the PB leaves.^[16] In conclusion, the ethanolic extract of PB was found to have various phytochemical compounds.

Cytotoxicity effects of BG, PB, and BGPB-conditioned media on DPSC

BG-conditioned media maintained over 70% DPSC cell viability across all concentrations except 6 mg/mL, with a steady increase in proliferation from days 1 to 7. This effect is likely due to BG's ion-releasing capability, dissolving calcium, phosphorus, silicon, sodium, and strontium, which promote cell proliferation within 24 h.^[24] This finding was in agreement with other studies which showed that lower concentrations of BG promoted higher cell viability compared to higher concentrations of BG.^[24-26] BG at a concentration of 4 mg/mL showed the highest DPSC cell viability from day 1 to 7 and was significantly higher ($P < 0.05$) compared to the control on days 4 and 7. Thus, this concentration was concluded as the optimal dose for DPSC proliferation in this study.

DPSC treated with PB-conditioned media displayed high proliferation at all concentrations except 250 µg/mL and 500 µg/mL, which were consistently cytotoxic ($P < 0.05$) throughout the incubation period. These findings align with Majumdar and Subramanian,^[27] who reported that PB promoted mouse fibroblast proliferation in a dose-dependent manner. In contrast, PB was found to be cytotoxic toward squamous cell carcinoma (KB-cell lines),^[28] breast cancer (MCF-7), cervical cancer (HeLa), and lung cancer (SK-LU-1).^[29] This does not imply that PB is inherently toxic but rather suggests its potential antitumor and anticancer properties.^[30]

PB-conditioned media at a dose of 31.25 µg/mL had the strongest influence on DPSC proliferation, likely due to the effects of hydroxychavicol and 3-allyl-6-methoxyphenol (chavibetol) found in the PB extract. Zamakshshari *et al.*^[15] identified hydroxychavicol as an active compound with antioxidant properties, which can reduce oxidative stress, enhance stem cell survival, and promote proliferation and differentiation.^[23] Although no studies specifically examine PB's effects on DPSC proliferation, PB extracts have been shown to stimulate dermal fibroblast and umbilical cord-derived mesenchymal stem cell (UC-MSCs) proliferation at specific doses.^[7,31] The study by Thi *et al.* evaluated the effects of PB extracts on fibroblasts and UC-MSCs using a scratch assay. At concentrations of 0.025 µL/mL and 0.03 µL/mL, the extracts promoted fibroblast proliferation and enhanced UC-MSC growth. In contrast, at 0.058 µL/mL, the extract exhibited cytotoxic effects on both UC-MSCs and fibroblast cell lines, leading to cell death. Similarly, Hoang *et al.* investigated the effects of PB extract on fibroblasts isolated from umbilical cord stem cells and demonstrated that the extract stimulated cell proliferation. However, at a concentration of 0.06 µL/mL, the extract inhibited proliferation and exhibited cytotoxic effects. The optimal concentration for promoting fibroblast proliferation was 0.025 µL/mL.^[7]

The combination of BG and PB did not show a significant increase in DPSC proliferation compared to the control. The initial decrease in cell viability on day 1 in the BGPB group may reflect the cells' adaptation to the new microenvironment formed by the combined culture media and extract. Notably, BGPB at 4.00 mg/30.00 µg promoted higher cell proliferation than the control on days 4 and 7, suggesting an optimal concentration for cell growth. By day 14, DPSC metabolic activity declined, likely due to nutrient depletion and contact inhibition resulting from increased cell density. Despite the reduced proliferation observed in PB-only and BGPB groups, DPSC viability remained above 70%, indicating that these concentrations were not cytotoxic and warrant further investigation.

Determining the chemical compound of PB, BG, and BGPB-conditioned media

The study suggests that compound interactions in these media may trigger ion exchange processes, potentially forming new chemical compounds. Notably, oxime-, methoxyphenyl- was detected in both BG and PB-conditioned media, likely resulting from interactions with DMEM culture media, which contains essential and non-essential amino acids that may produce oximes involved in amino acid oxidation.^[32] Oximes, with diverse chemical structures, have demonstrated therapeutic potential, including antimicrobial, anti-inflammatory, antioxidant, and anticancer effects.^[33] In addition, 2-hydroxy-gamma-butyrolactone, known for its antioxidant, analgesic, antibacterial, and antifungal activities, was detected in all three samples. The major compound of PB-conditioned media was also detected in other medicinal plants such as *Amygdalus spinosissima*^[34] and *Crotalaria paniculata*.^[35]

In BGPB-conditioned media, 2,4-di-tert-butylphenol showed the highest retention area, followed by octadecanoic acid, 2,3-dihydroxypropyl ester. A lipophilic phenol, 2,4-di-tert-butylphenol, is found in various plants, including sweet potato and pomegranate, and reduces apoptosis by mitigating hydrogen peroxide-induced oxidative stress in PC12 cells.^[36] Conversely, it has demonstrated anticancer properties against several cell lines, including MCF-7.^[37] Octadecanoic acid, 2,3-dihydroxypropyl ester, detected in *Gymnopilus junonius* mushrooms and *Cenchrus biflorus Roxb.* leaves, has shown antimicrobial and anticancer activities.^[38]

CONCLUSION

This *in vitro* study analyzed the phytochemical composition of PB extract and the cytotoxic effects of BG, PB, and BGPB on DPSCs. The ethanolic PB extract was rich in phenolics, particularly hydroxychavicol (70.37%) and chavibetol (17.78%). BG promoted DPSC proliferation at 4 mg/mL,

while cytotoxicity was observed at 2 mg/mL. PB promoted DPSC proliferation at 31.25 µg/mL, with cytotoxic effects at 250 µg/mL and 500 µg/mL. The BG-PB combination (4 mg/30 µg) was identified as the best proliferative response. BG, PB, and BGPB-conditioned media revealed the presence of active compounds such as phenolics, terpenoids, esters, fatty acids, ketones, furans, imines, siloxanes, and amides.

Ethical approval: This study was reviewed by the Human Research Ethics Committee, Universiti Sains Malaysia (JEPeM USM), and was granted an exemption from ethical review (Reference No.: USM/JEPeM/21080553; dated: 1st September 2021).

Declaration of patient consent: Patient's consent is not required as there are no patients in this study.

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