ORIGINAL ARTICLE

Dental Pulp Stem Cells Response to Chrysanthemum Flower Extract

Nurul Shazwani Mohd Zain[1], Nik Syahirah Aliaa Nik Sharifulden[1], Siti Noor Fazliah Mohd Noor[1], Nurulakma Zali[1], Navaneetha Cugati[2], Nur Fatihah Nordin[3]

[1] Craniofacial and Biomaterials Sciences Cluster, Advanced Medical & Dental Institute, Universiti Sains Malaysia, Bertam, 13200 Kepala Batas, Pulau Pinang, Malaysia
[2] Department of Paedodontics and Preventive Dentistry, Coorg Institute of Dental Sciences, Virajpet, 571218, Coorg Institute, Karnataka State, India
[3] Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia

ABSTRACT

Introduction: *Chrysanthemum rubellum (durian)* flower (CR) is well-known for its usefulness in conventional and advanced medicine. Bioactive glass properties are geared towards hard tissue regeneration. Hence, this study aims to investigate the response of dental pulp stem cells (DPSC) when exposed to bioactive glass-chrysanthemum flower extract-conditioned medium. **Methods:** *Chrysanthemum rubellum (durian)* yellow coloured petals freeze dried extracts (CRE) was prepared by separation and agitation in distilled water where the final powdery compound was being investigated and combined with melt-derived BG 45S5 powder to produce BGCRE-conditioned medium. The CRE extracts in various concentrations and BGCRE-conditioned medium were exposed to DPSC and the cells responses were assessed using AB and MTT assays. The CRE and BGCRE-conditioned media were also assessed using ICP-OES to check for ionic release profile from both medium. **Results:** The CRE-conditioned medium (7.81, 15.63, 31.25 and 62.5 µg/ml) showed a dose-dependent effect towards DPSC from Days 1 until 14. The BGCRE-conditioned medium containing BG powders (1 mg/ml) with the lower amount of CRE extracts (0.02 and 0.1 mg/ml) promoted DPSC viability and proliferation rate from Days 1 until 14 based on AB and MTT assays. The BGCRE-conditioned medium has potentially affected the DPSC viability and proliferation. **Conclusion:** The presence of CRE in BGCRE-conditioned medium enhanced the DPSC viability and proliferation possibly through the combined effect of CRE and BG. This BGCRE combination showed potential as natural medicament for dental tissue regeneration.

Keywords: Bioactive glass, *Chrysanthemum rubellum*, Dental pulp stem cells, Biocompatibility

Corresponding Author:
Siti Noor Fazliah Mohd Noor, PhD
Email: fazliah@usm.my
Tel: +6013-3960943

INTRODUCTION

Chrysanthemum rubellum (durian) flower (CR) is one of the species in Chrysanthemum broad family and is one of the most well-known for its usefulness around the entire globe and progress widely in both conventional and advanced medicine. Generally, aside from being produced as herbal drinks that provide soothing, refreshing and body-cooling effects, this perennial herb has been widely marketed for treating various inflammatory-related diseases with high efficacy and low toxicity. Numerous researches proved that *Chrysanthemum rubellum* extract (CRE) possess anti-inflammatory (1,2), anti-cancer (3), anti-osteoporosis and antioxidant (4), and antimicrobial properties (5). The phytochemical components of CRE include 1,3-Butanediol (6), Undecane (7), Benzene, 1,3-bis(1,1-dimethylethyl) (8), Eicosane (9,10) and Phenol, 2,4-bis(1,1-dimethylethyl) (11). The bioactive glass composition based on 45S5 Bioglass® is capable of forming an interactive bonding to bone host tissues as well as serving as a platform for osteogenic stem cells able to attach and differentiate in physiological environments (12). The golden commercialised constituent of 45S5 which comprises of 45% SiO_2, 24.5% CaO, 24.5% Na_2O and 6% P_2O_5 (in weight percentage, wt.%) creates an exchange of its critical soluble components in physiological environment such as simulated body fluid (SBF) due to the reactions occur on the material surface and subsequently lead to positive intracellular and extracellular responses, creating a silica-rich layer and deposition of apatite-layer on the glass surface (13,14). Bioactive glass (BG) can be synthesised via two main routes, namely melt derived and sol-gel. Melt derived technique is a conventional way to fabricate BG 45S5 via heating the precursor materials at a very high temperature and subsequently, the molten liquid undergoes rapid quenching for obtaining glass frits. Melt derived technique is in favour of producing BG as it is

likely to be fabricated in large scale and of high quality amorphous BG powder (15). It has been more than a century that orthopaedics needs to face challenges more than treatments on bones' infectious diseases; mostly due to accidents or age-related ailments that involves a greater bone repair (16). On top of that, as life expectancy increases, the needs to ensure prolongation of device survivability in physiological environment has led to many crucial studies as many believe that the whole concept of repairing should be shifted from replacement to regeneration of human tissues especially bone (17). Ensuring the ability of stem cells targeted for BG's attachment, differentiation and proliferation is a very crucial part in bone regeneration as well as ensuring a protective effect over the physiological surrounding, as in the whole structure must be able to perform the bioactivity effectively with no toxicity possibilities. Therefore, our targeted cells, dental pulp stem cells (DPSC) are known to be highly proliferative, multipotent, able to induce mineralised nodule in monolayer culture and producing dentine-like regenerative tissue similar to bone (18-20). Thus, this study aims to determine the effectiveness of hybrid medium between BG and CRE at different concentrations towards DPSC's proliferation whilst inducing a protective environment. The CRE effects on DPSC has not been widely reported, hence, this study is important to provide current knowledge regarding the benefits of CRE in assisting cells proliferation while providing necessary protections over DPSC and its benefits which may be similar to other reported studies with different kinds of Chrysanthemum family such as *Chrysanthemum indicum* and *Chrysanthemum zawadskii*.

MATERIALS AND METHODS

Extraction of Chrysanthemum Rubellum (Durian) Flower Extract (CRE)

Chrysanthemum rubellum (durian) (CR) fresh flowers were obtained from a farm in Cameron Highland, Malaysia. The species used namely *Chrysanthemum rubellum (durian)* flower extract (CRE) were confirmed, specified and indexed (11548) at Herbarium Unit, USM Penang, Malaysia. CRE was firstly prepared by separating petals from peduncle. Then, the petals were washed twice in distilled water, and air dried for 20 min and further dried in clean air oven at 40 °C for 48 h. The dried petals were then grounded coarsely by using an electric blender until it reached powdery state. By using the ratio of 50 g:1000 ml of CRE powder and distilled water, the mixture was then continuously agitated on a shaking water bath for 24 h at 40 °C. The liquid mixture was then filtered and centrifuged at 1500 rpm for 10 min at 25 °C. The filtered residue was freeze dried at -50 °C for 7 days and subsequently stored at -20 °C until further usage.

Fabrication of melt derived bioactive glass (BG)

Raw materials used in the fabrication of BG 45S5 were SiO_2 (Sigma-Aldrich, 14808-60-7), $CaCO_3$ (Merck, 471-341-1), Na_2CO_3 (Sigma-Aldrich, 497-19-8) and P_2O_5 (Sigma-Aldrich, 1314-56-3). The bioactive glass 45S5 is prepared as $45SiO_2$-24.5CaO-$24.5Na_2O$-$6P_2O_5$ in weight percentages (wt.%). The raw materials powders were mixed overnight at room temperature with 25 rpm for homogeneity.

The BG powders were melted in a platinum crucible at 1400±10 °C with soaking time for 90 min. Molten glass was rapidly quenched into distilled water to prevent crystallisation in order to obtain frits, and the glass frits were dried overnight at 110 °C in an oven. The frits were then milled by using planetary mill (Fritch Pulverisette) for 10 min at 500 rpm. Powders from the batch was sieved for 30 min in a mechanical shaker (Fritsch) to obtain particle size less than 38 µm prior to cell culture studies. The BG powders were characterised as previously reported (21).

High Performance Liquid Chromatography (HPLC) analysis

The CRE extract was analysed using the HPLC system (Agilent Technologies, United States). A Zorbax eclipse xdb-c18 (4.6 mm x 250 mm, 5 µm) column was used for the separation of gallic acid, luteolin, caffeic acid, chlorogenic acid and quercitrine. Stock solutions of gallic acid (Sigma Aldrich, 149-91-7), luteolin (HWI Analytik GmbH, 491-70-3), caffeic acid (Sigma Aldrich, 331-39-5), chlorogenic acid (HWI Analytik GmbH, 327-97-9) and quercitrine (HWI Analytik GmbH, 522-12-3) were prepared with the concentration of 0.1 mg/ml (100 ppm). The CRE were weighted on a weighing boat and dissolved in methanol (HPLC grade) with the concentration of 100 mg/ml. Solution for standard and sample was filtered using 0.22 µm syringe filter before being analysed with HPLC system. The mobile phases used were 2% aqueous acetic acid as solvent A and acetonitrile which contained 2% acetic acid as solvent B. There were 3 gradient elution program namely initial A-B (99.5:0.5); in 40 min A-B (80:20); in 20 min A-B (60:40); in 2 min back to initial conditions; in 15 min conditioning of the column. The flow rate used was 1 ml/min and the chromatograms were recorded at 280 nm.

Gas Chromatography – Mass Spectrometry (GCMS) analysis

The volatile compounds were extracted by preparing 2 g of filtered CRE petal extract samples together with 1 ml of internal standard reference methanol (MeOH). These mixtures were dissolved and placed in the sample extraction column, and analysed via GC-MS. A coupled GC-MS system comprises of Agilent 7890A gas-chromatograph (GC) system and a 5975C mass spectrometer (MS) was used to analyse volatile compounds within the CRE mixture. The HP-5MS (30 m length x 0.25 mm ID, 0.25 µm film thickness) analytical fused capillary column was used for chromatographic

separations and 1 µl of CRE sample was injected into the injector port held at 280 °C. During these overall processes, the injector temperature was first held at 50 °C, and then gradually increased to 280 °C at the rate of 4 °C/min, maintained for 5 min and the total run time is 62.5 min. The helium carrier gas flow rate was at 1 ml/min. Meanwhile, mass spectrum in the splitless mode was generated at 71 eV and ion source/ injector temperature at 280 °C. The total ion currents which proportional to the chromatograms were monitored and recorded in 30-800 mass-range. The volatile components were quantitatively determined by matching the retention index (RI) and mass spectra with the standard library values. The relative percentage of each extract constituent was expressed as percentage with peak area normalisation.

Cell culture studies

Dental pulp stem cells (DPSC, Lonza, USA) were grown and maintained in DMEM cell culture media supplemented with foetal bovine serum (FBS, 10% v/v) and Antibiotic/Antimycotic (A/A, 1% v/v). When DPSC reached 80-90% confluence, the cells were seeded in the 96-well plates with a seeding density of 5 X 10^3 cells/cm^2. Then, the cells were exposed with the conditioned medium, and the medium was changed every 48 h.

CRE-conditioned medium preparation

The CRE freeze dried powders were weighted and incubated with the culture medium for different concentrations (7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1000 and 2000 µg/ml) in an incubator shaker for 4 h at 37 °C. Then, the CRE-conditioned medium was filtered using 0.22 µm syringe filter and supplemented with 1% A/A and 10% FBS followed by incubation in a CO_2 incubator at 37 °C overnight prior to use on cells.

BGCRE-conditioned medium preparation

The CRE and BG powders were weighted and the combination of BG powders (1 mg/ml) and CRE extracts (0.02, 0.1 and 1 mg/ml) were prepared with culture medium and incubated in an incubator shaker for 4 h at 37 °C. Then, the BGCRE-conditioned medium was filtered using 0.22 µm syringe filter. The conditioned medium was supplemented with 1% A/A and 10% FBS followed by incubation at 37 °C in a CO_2 incubator overnight prior to use on cells.

Cell viability assessment using AlamarBlue

The DPSC were seeded in a 96-well plate with a seeding density of 5 X 10^3 cells/cm^2 and treated with CRE- and BGCRE-conditioned media followed by incubation in a CO_2 incubator at 37 °C with 5% CO_2. At designated days (1, 4, 7 and 14), the conditioned medium was removed and cells were washed with 100 µl of DPBS (Gibco, USA). Then, 150 µl of 10% (v/v) AlamarBlue in DMEM with no phenol red (Gibco, USA) was added per well (including one well with no cells as blank). After 2 h of incubation at 37 °C, 100 µl of the reaction product was then transferred to a black Costar 96-well plate, and the AB fluorescence was read at excitation wavelength of 544 nm and emission at 590 nm using a microplate reader (FluoStar Omega, BMG).

Cell cytotoxicity assessment using MTT assay

The DPSC were treated with BGCRE-conditioned medium and incubated at 37 °C in an incubator. At designated days (1, 4, 7 and 14), the BGCRE-conditioned medium was removed and 5 mg/ml MTT salt solution (M9464, Invitrogen, USA) was added into each well including one with no cells to be used as blank. Then, the cells were incubated in an incubator for 4 h at 37°C followed by removing the medium from the wells and addition of 100 µl DMSO. The absorbance was measured using a microplate reader (Fluostar Omega BMG Labtech, Germany) at a wavelength of 570 nm with a reference wavelength of 620 nm.

Ion dissolution from BG and CRE in culture media

The BG powders (1 mg/ml), CRE (0.1 mg/ml) and the combination of BG powders (1 mg/ml) and CRE (0.1 mg/ml) were weighted on a weighing boat based on the powder to liquid ratio. Then, the BG powders and CRE were incubated in culture media using the conical sterile polypropylene centrifuge tubes and sealed with paraffin film. The conditioned medium was incubated in the incubator shaker at 37 °C with 100 rpm (Ika Ks 4000, USA). After 30, 60, 120, and 240 min, 10 ml of solution were collected from the centrifuge tubes and filtered using 0.22 µm syringe filter followed by keeping the solution at -80 °C freezer prior to ICP-OES test.

Inductively Coupled Plasma – Optical Emission Spectrometer (ICP–OES)

Briefly, the collected solutions containing dissolution ions from BG and CRE were diluted by a factor of 10 in ultrapure water. Then, the elemental concentrations of silicon (Si), calcium (Ca), sodium (Na) and phosphorus (P) ions were measured using ICP-OES machine (Perkin Elmer, USA). Standard calibration curves were obtained by preparing standard solutions containing Si Ca, Na and P. Three replicates were measured for each element.

pH evaluation

At interval time points (0, 30, 60, 120 and 240 min) pH of the culture media containing BG powders, CRE included with the combination of BG powders and CRE were evaluated using a calibrated pH meter (Mettler Toledo, USA).

Statistical analysis

Data of the experiments are represented as mean ± standard deviations (Mean±SD), obtained from two independent experiments and also prepared in quadruplicate. The data were analysed using One-way ANOVA with *Scheffe* post-hoc test. This test compared DPSC exposed to all conditioned medium with the control medium on each time interval points, and

p-values <0.05 is being considered as significant.

RESULTS

GCMS

Forty-six volatiles compounds were identified in the CRE sample via GC-MS analysis and the chemical compositions are presented in Table 1. There are 9 major components detected which are crucial phytocompounds of CRE.

Table I: Chemical compositions of vital components in CRE detected via GC-MS analysis

Number	Components	R.t.[a]	Percentage (%)[b]
1	1,3-Butanediol, (S)-	3.889	78.87
2	Undecane	10.976	0.77
3	Benzene, 1,3-bis(1,1-dimethylethyl)-	16.127	0.26
4	Eicosane	23.617	0.21
5	Phenol, 2,4-bis(1,1-dimethylethyl)	24.322	0.32
6	Tetratetracontane	29.857	0.42
7	7,9-Di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione	35.212	0.39
8	Hexadecanoic acid, methyl ester	35.398	0.50
9	Dibutyl phthalate	36.258	1.73

[a]Retention time (min).
[b]Relative percentage calculated by integrated peak area in Agilent MSD Chemstation data analysis program.

HPLC

The fingerprinting of HPLC for CRE is illustrated (Fig. 1). Identification of flavonoids and phenolic compounds was based on the chromatic behaviour based on standard references and also comparison with those in literature. Several trials were attempted for adjusting the flow rate, gradient profile and wavelength. The compounds within CRE were well separated and classified into two groups namely phenolic acid and flavonoids as shown in Table II. Presence of phenolic acids (gallic acid, chlorogenic acid and caffeic acid) and flavonoids (quercitrine and luteolin) in the CRE extract based on the fingerprinting of HPLC as shown in Fig. 1 and Table 3. Cholorogenic acid (tr=21.04 min; peak 2) and luteolin (tr=46.12 min; peak 5) was detected in the CRE extract. Gallic acid (tr=5.88 min; peak 1), caffeic acid (tr=24.08 min; peak 3) and quecitrine (tr=42.93 min; peak 4) were also present in the CRE extract. However, the CRE contained less amount of quecitrine based on the height of the compound which was 52.4 mAU and presence of quercitrine (peak 4) cannot be seen clearly (Fig. 1).

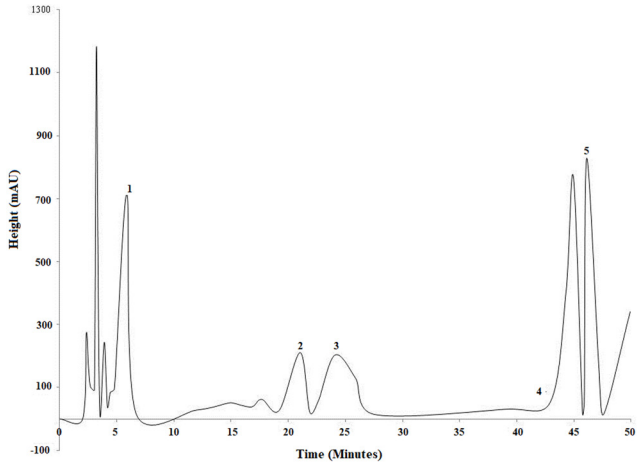

Figure 1: High performance liquid chromatography (HPLC) profile for Chrysanthemum rubellum (durian) Flower Extract (CRE) at 280 nm. Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), quercitrine (peak 4) and luteoline (peak 5).

Table II: Compounds of CRE based on HPLC analysis

No.	Compounds	Retention time (t_r, minutes)	Height (mAU)
	Phenolic acids		
1	Gallic acid	5.88	713.6
2	Chlorogenic acid	21.04	211.0
3	Caffeic acid	24.08	202.8
	Flavonoids		
4	Quecitrine (4)	42.93	52.4
5	Luteolin (5)	46.12	828.6

Cell culture studies

Response of DPSC towards CRE-conditioned medium

AlamarBlue assays in Fig. 2 represented the viability of DPSC exposed to CRE-conditioned medium. The DPSC viability increased throughout Days 1 until 14. DPSC showed higher proliferation rate when exposed to lower powder to liquid ratio of CRE-conditioned medium at dose of 7.81, 15.62, 31.25 and 62.50 mg/ml. However, the cell proliferation rate was lower starting from Days 1 until 14 for the higher powder to liquid ratio for CRE-conditioned medium at dose of 125, 250, 500, 1000 and 2000 mg/ml.

Response of DPSC towards BGCRE-conditioned medium

AlamarBlue (Fig. 3a) and MTT (Fig. 3b) assays showed the viability and proliferation rate of DPSC upon exposure to BGCRE-conditioned medium. DPSC exposed to BGCRE-conditioned medium showed different responses due to various amount of CRE added to BG. The MTT assay was also used as a measure of cytotoxicity of the BGCRE-conditioned medium towards DPSC by addressing the percentage level of toxicity towards the cells responses,

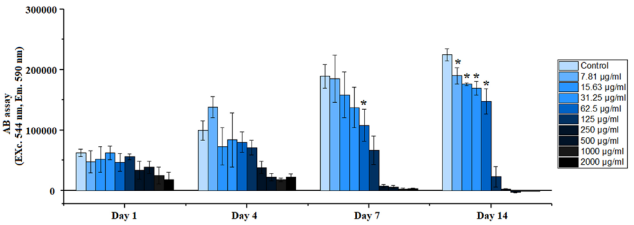

Figure 2: The viability of DPSC assessed using AlamarBlue assay upon exposure to CRE-conditioned media. *denotes a significant of the marked bar compared to DPSC incubated with control at the same time point.

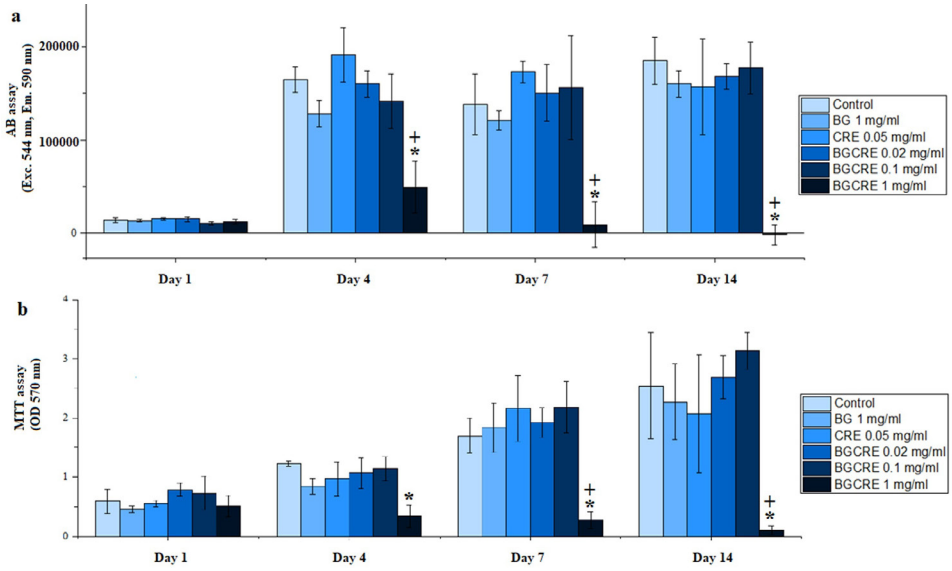

Figure 3: DPSC response towards BG CRE-conditioned medium. (a) The cell viability assessment of DPSC assessed using Alamar Blue assay. *denotes a significant of the marked bar compared with CRE 0.05 mg/ml. +denotes a significant of the marked bar compared with BG 1 mg/ml. (b) The cytotoxicity assessment of DPSC assessed using MTT assay. *denotes a significant of the marked bar compared with CRE 0.05 mg/ml. +denotes a significant of the marked bar compared with BG 1 mg/ml.

however, in the current study, MTT results are presented in values of optical density (OD) for ease of clarification and comparison towards AB assay. The viability and proliferation rate of DPSC for BGCRE-conditioned medium at 0.02 and 0.1 mg/ml concentrations were higher until Day 4 and remain constant until Day 14. DPSC responded favourably towards lower powder to liquid ratio of BG (1 mg/ml) (Fig. 3a) but when BG powder at 1 mg/ml was combined with CRE at concentration of 1 mg/ml, the viability and proliferation rate decreased due to the higher powder to liquid ratio of CRE as shown in Fig. 3a which is toxic towards the cells. The DPSC proliferation rate showed an increasing trend from Days 1 until 14. The combined dose of both BGCRE-conditioned medium at 1 mg/ml was toxic to DPSC with decreasing viability and proliferation as shown in AlamarBlue (Fig. 3a) and MTT (Fig. 3b) results. DPSC exposed to lower dose of BGCRE-conditioned medium at 0.02 and 0.1 mg/ml showed statistically significant higher proliferation trends compared to control media and CRE with dose of 0.05 mg/ml, and with the presence of BG at dose of 1 mg/ml. Presence of CRE in BGCRE-conditioned medium significantly enhances the DPSC proliferation rate compared to DPSC exposed solely to CRE-conditioned media. Hence, it can be seen that higher BG dose and higher CRE dose is cytotoxic to DPSC leading to lower cell viability and proliferation.

Ionic dissolution studies

The silicon (Si) ions in BGCRE dissolute faster than silicon ions in BG upon immersion in DMEM culture media (Fig. 4(a)). There is no silicon released from CRE incubated in DMEM throughout the 240 min. However, CRE enhanced dissolution rates of silicon when combined with BG in BGCRE-conditioned medium compared to BG-conditioned medium alone. The sodium (Na) dissolution rate (Fig. 4(b)) was different for BG, CRE and BGCRE. Sodium released from BGCRE was lower for the first 30 min, then became higher at 120 min which subsequently decreased at 240 min. Sodium

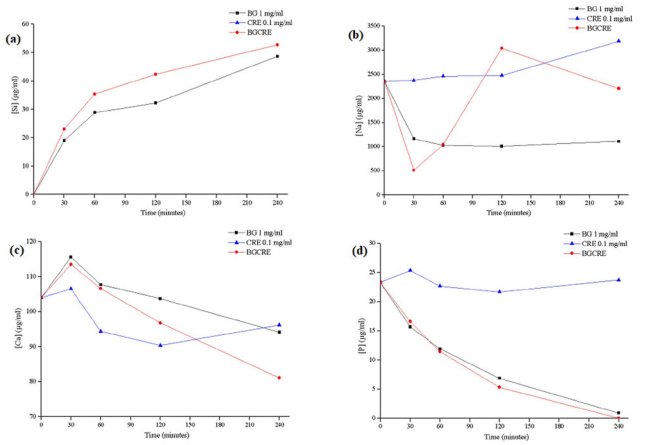

Figure 4: Ion dissolution profiles released from BG, CRE and BGCRE in DMEM culture medium for (a) Si (b) Na (c) Ca and (d) P ions.

released tend to increase over time in CRE-conditioned medium compared to BG-conditioned medium. Calcium ions dissolute faster in BGCRE-conditioned media compared to CRE and BG-conditioned media (Fig. 4(c)). Meanwhile, phosphorus ions (Fig. 4(d)) dissolution were faster in BG and BGCRE-conditioned medium that tend to be almost nil upon reaching 240 min. However, phosphorus ions released from CRE-conditioned media remain stable over 240 min.

pH evaluation

The pH values showed an increasing trend over time for BG-, CRE- and BGCRE-conditioned media in DMEM is shown in Fig. 5. BGCRE-conditioned medium showed the highest pH value compared to the BG- and CRE-conditioned media. The CRE-conditioned medium showed the lowest pH values (8.08) compared to the BG- (8.80) and BGCRE-conditioned media (8.9). The pH behaviour of BG- and BGCRE- conditioned medium showed similar trends but the BGCRE-contitioned medium pH values were higher than BG-contitioned medium. Hence, presence of CRE within BG may enhance the pH values in the solution over 240 min.

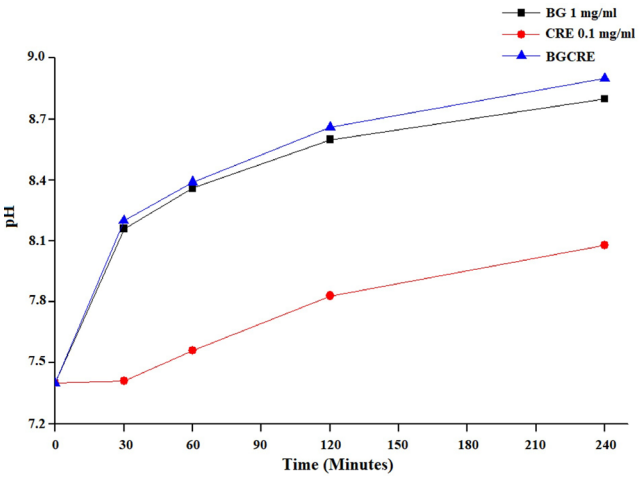

Figure 5: pH of the DMEM solution after immersion of BG, CRE and BGCRE

DISCUSSION

Based on GC-MS analysis, among the volatiles detected, the most abundant volatile found is 1,3-Butanediol, (S)-, an ethanol dimer, comprises around 78.87% of the overall sample where this principal component was previously reported as a useful cryoprotectant against hypoxia via a cerebral protective effect mediated by brain ketone metabolism when it is being converted to Я-hydroxybutyrate in physiological environment (7). Second vital component detected is Undecane, a very well-known alkane for its antimicrobial property (8). Benzene, 1,3-bis(1,1-dimethylethyl)- is another compound that is vital for its antibacterial activity (9). Eicosane is particularly emphasise on its antifungal and antibacterial characteristics (10,11). Phenol, 2,4-bis(1,1-dimethylethyl) phytocompound is responsible for activation of antioxidant and antibacterial activities where it showed antibacterial activation of Mango kernel towards gram positive bacteria (12), antioxidant property of phenol, 2,4-bis(1,1-dimethylethyl) as compared to L-ascorbic acid in Pinus granatum (pomegranate) rind extract (22) and the correlation of antibacterial and antioxidant properties of phenol, 2,4-bis(1,1-dimethylethyl) due to its inhibition towards reactive oxygen species (ROS) production in both Aspergillus and Phytophthora cinnamon, thus subsequently control the production of hydrogen peroxide in avocado roots (23). Thus, CRE contain abundant volatiles compound suggestive that these significant bioactive phytochemicals-rich flower has many advantages and pharmaceutical benefits. Detection of cholorogenic acid and luteolin in the CRE extract reflects the compound present in *chrysanthemum indicium* flower (CIF), also a well-known Chrysanthemum species (24). Gallic acid, caffeic acid and quecitrine were also present in other herbs such as Cretan barberry (25) and Scutia buxifolia (26).

The release of silica ions from the BG and BGCE indicated the first stage of dissolution through the breaks of Si–O–Si bond during first stage of HCA mechanism (27). Decreasing of calcium and phosphorus ions followed by increasing in silicon ions contributed to the formation of CaP layer (28). After 240 min soaked in DMEM solutions, BGCRE showed almost similar trends with BG for all ion's dissolution profile. Thus, presence of 0.1 mg/ml CE inhibits the formation of CaP layer after combination with the BG when soaked in DMEM solution. Dissolution rates of BGCRE are faster compared to dissolution rates of BG in DMEM solution. BG exhibit lower dissolution rate even though the amount of BG concentration were similar for BG and BGCRE which was 1 mg/ml. Presence of CRE in BGCRE promoted and enhanced dissolution rate to become faster. Rate of apatite formation on the BG surface become slower due to the slower dissolution rate (27). Therefore, rate of apatite formation is faster from BGCRE due to the presence of CRE that caused faster dissolution rate based on ICP-OES analysis. Higher pH values indicate higher BG degradation and hence, its bioactivity would tend to be higher (29). Thus, bioactivity of BGCRE is higher compared to BG bioactivity as proved by the higher dissolution rate.

The time frame for DPSC exposure towards BG- and BGCRE-conditioned medium was chosen at 14 days to highlight the time needed for initial mineralisation of DPSC towards osteogenic lineages (20), however, these data are not available and further studies should look into genetic changes that may have occurred towards DPSC. Studies shown that BG are able to change genetic expression of DPSC towards osteoblastic lineages (19), hence the addition of CRE within the BG need to be explored further for CRE properties that may have enhance and trigger the DPSC responses towards osteogenic lineages and genetic changes within the cells itself.

CONCLUSION

Based on this study, it can be concluded that 0.1 mg/ml CRE was the optimal dose for doping with the bioactive glass powders. The GC-MS and HPLC analyses showed the presence of crucial volatile phytocompounds and presence of flavonoids and phenolic acids respectively in CRE. Presence of CRE within bioactive glass powder able to promote the bioactive glass dissolution rate subsequently contributed to promoting DPSC viability and proliferation rate from Days 1 to 14 especially at lower amount of bioactive glass powder to liquid ratio namely 7.81, 15.63, 31.25 and 62.5 mg/ml for CRE-conditioned medium promoted DPSC viability based on AlamarBlue assay. The BGCRE-conditioned medium containing BG powders (1 mg/ml) with the lower amount of CRE powders (0.02 and 0.1 mg/ml) also enhanced DPSC viability and proliferation rate based on AlamarBlue and MTT assays suggesting that these combinations are not toxic to DPSC. Presence of CRE promoted ionic dissolution rate from BGCRE-conditioned medium based on ICP-OES analysis, hence

promoting BGCRE bioactivity based on increasing pH values. Further studies on different characterisation and method of CRE extraction is encouraged as this Chrysanthemum species used has shown to assist BG in enhancing proliferation of dental pulp stem cells, not to mention the benefits exhibited by this species in providing protective physiological environment for dental hard tissue regeneration.

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REFERENCES

1. Dong M, Yu D, Duraipandiyan V, Abdullah Al-Dhabi N. The protective effect of Chrysanthemum indicum extract against ankylosing spondylitis in mouse models. Biomed Research International. 2017;8206281(7).
2. Wu X-L, Li C-W, Chen H-M, Su Z-Q, Zhao X-N, Chen J-N, et al. Anti-inflammatory effect of supercritical-carbon dioxide fluid extract from flowers and buds of Chrysanthemum indicum Linnen. Evidence-Based Complementary and Alternative Medicine. 2013; 2013:413237(13).
3. Kim C, Kim MC, Kim SM, Nam D, Choi SH, Kim SH, et al. Chrysanthemum indicum L. extract induces apoptosis through suppression of constitutive STAT3 activation in human prostate cancer DU145 cells. Phytotherapy Research. 2013; 27(1):30-38.
4. Luyen BTT, Tai BH, Thao NP, Lee YM, Lee SH, Jang HD, et al. The Anti-Osteoporosis and Antioxidant Activities of Chemical Constituents from Chrysanthemum indicum Flowers. Phytotherapy Research. 2015;29(4):540-548.
5. Arokiyaraj S, Arasu MV, Vincent S, Prakash NU, Choi SH, Oh Y-K, et al. Rapid green synthesis of silver nanoparticles from Chrysanthemum indicum L and its antibacterial and cytotoxic effects: an in vitro study. International Journal of Nanomedicine. 2014;9:379-388.
6. Marie C, Bralet A-M, Bralet J. Protective action of 1, 3-butanediol in cerebral ischemia. A neurologic, histologic, and metabolic study. Journal of Cerebral Blood Flow and Metabolism. 1987;7(6):794-800.
7. Krishnamoorthy K, Subramaniam P. Phytochemical profiling of leaf, stem, and tuber parts of Solena amplexicaulis (Lam.) Gandhi Using GC-MS. International Scholarly Research Notices. 2014; 2014;567409(13).
8. Dehpour A, Yousefian M, Kelarijani SJ, Koshmoo M, Mirzanegad S, Mahdavi V, et al. Antibacterial activity and composition of essential oils of flower Allium rotundum. Advanced Environmental Biology. 2012;6:1020-5.
9. Ahsan T, Chen J, Zhao X, Irfan M, Wu Y. Extraction and identification of bioactive compounds (Eicosane and Dibutyl phthalate) produced by Streptomyces strain KX852460 for the biological control of Rhizoctonia solani AG-3 strain KX852461 to control target spot disease in tobacco leaf. AMB Express. 2017;7(1):54.
10. Asha K, Priyanga S, Hemmalakshmi S, Devaki K. GC-MS Analysis of the Ethanolic Extract of the whole Plant Drosera indica L. International Journal of Pharmacognosy and Phytochemical Research. 2017; 9(5); 685-688.
11. Abdullah A-SH, Mirghani MES, Jamal P. Antibacterial activity of Malaysian mango kernel. African Journal of Biotechnology. 2011;10(81):18739-18748.
12. Bahniuk MS, Pirayesh H, Singh HD, Nychka JA, Unsworth LD. Bioactive glass 45S5 powders: effect of synthesis route and resultant surface chemistry and crystallinity on protein adsorption from human plasma. Biointerphases. 2012;7(1):41.
13. Gerhardt L-C, Boccaccini AR. Bioactive glass and glass-ceramic scaffolds for bone tissue engineering. Materials. 2010;3(7):3867-3910.
14. Jones JR. Review of bioactive glass: from Hench to hybrids. Acta Biomaterialia. 2013;9(1):4457-4486.
15. Pirayesh H, Nychka JA. Sol–Gel Synthesis of Bioactive Glass-Ceramic 45S5 and its in vitro Dissolution and Mineralization Behavior. Journal of The American Ceramic Society. 2013;96(5):1643-1650.
16. Lombardi M, Gremillard L, Chevalier J, Lefebvre L, Cacciotti I, Bianco A, Montanaro L. A comparative study between melt-derived and sol-gel synthesized 45S5 bioactive glasses. Key Engineering Materials; 2013:541:15-30. Trans Tech Publ.
17. Hench LL, Polak JM. Third-generation biomedical materials. Science. 2002;295(5557):1014-1017.
18. D'Aquino R, Papaccio G, Laino G, Graziano A. Dental pulp stem cells: a promising tool for bone regeneration. Stem Cell Reviews. 2008;4(1):21-26.
19. El-Gendy R, Yang XB, Newby PJ, Boccaccini AR, Kirkham J. Osteogenic differentiation of human dental pulp stromal cells on 45S5 Bioglass® based scaffolds in vitro and in vivo. Tissue Engineering Part A. 2012;19(5-6):707-715.
20. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences. 2000;97(25):13625-13630.
21. Ibrahim NF, Mohamad H, Noor SNFM. Characterization on melt-derived bioactive glass powder from SiO2-CaO-Na2O-P2O5 system. Journal of Non-Crystalline Solids. 2017;462:23-31.
22. Prakash A, Suneetha V. Punica granatum (Pomegranate) Rind extract as a potent substitute for L ascorbic acid with respect to the antioxidant activity. Research Journal of Pharmaceutical, Biological and Chemical Sciences. 2014;5(2):597-603.
23. Teresa R-CM, Rosaura V-G, Elda C-M, Ernesto G-P.

The avocado defense compound phenol-2, 4-bis (1, 1-dimethylethyl) is induced by arachidonic acid and acts via the inhibition of hydrogen peroxide production by pathogens. Physiological and Molecular Plant Pathology. 2014;87:32-41.
24. He J, Wu X, Kuang Y, Wang T, Bi K, Li Q. Quality assessment of Chrysanthemum indicum Flower by simultaneous quantification of six major ingredients using a single reference standard combined with HPLC fingerprint analysis. Asian Journal of Pharmaceutical Sciences. 2016;11(2):265-272.
25. Kukula-Koch W, Aligiannis N, Halabalaki M, Skaltsounis A-L, Glowniak K, Kalpoutzakis E. Influence of extraction procedures on phenolic content and antioxidant activity of Cretan barberry herb. Food chemistry. 2013;138(1):406-413.
26. Da Silva AR, Moreira LdR, Brum EdS, de Freitas ML, Boligon AA, Athayde ML, et al. Biochemical and hematological effects of acute and sub-acute administration to ethyl acetate fraction from the stem bark Scutia buxifolia Reissek in mice. Journal of Ethnopharmacology. 2014;153(3):908-916.
27. Sepulveda P, Jones J, Hench L. In vitro dissolution of melt-derived 45S5 and sol-gel derived 58S bioactive glasses. Journal of Biomedical Materials Research. 2002;61(2):301-311.
28. Srivastava AK, Pyare R. Characterization of ZnO Substituted 45S5 Bioactive glasses and glass-ceramics. Journal of Materials Science Research. 2012;1(2):207-220.
29. Vyas V, Kumar A, Singh S, Pyare R. Effect of nickel oxide substitution on bioactivity and mechanical properties of bioactive glass. Bulletin of Materials Science. 2016;39(5): 1355–1361.