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Enhancement of dental pulp stem cell viability using *Acmella oleraceae* extract combined with bioactive glass

***Abstract*—** This study aims to determine the effects of bioactive glass (BG) combined with *Acmella oleracea* (AO) extracts on dental pulp stem cells (DPSC) viability. DPSC were exposed to different combinations of BG-AO leave extract-conditioned medium. The BG 45S5 powder was synthesized using the sol-gel method. AO extract was prepared using ethanol extraction method. Gas Chromatography–Mass Spectrometry (GCMS) analysis of the AO ethanol extract was performed on a GCMS system consisting of an Agilent 6890 gas chromatograph coupled with an Agilent 5973 mass spectrometer. Sol-gel BG conditioned medium doped with AO extracts at various concentrations (25, 50, 100 and 250 µg/mL) with BG (1 mg/ mL) were prepared and exposed to DPSC. The DPSC was also treated using BG- and AO- only conditioned medium and non-treated cell as control. The DPSC cells' responses were assessed using Alamar Blue (AB) assay. The results showed that GCMS analysis revealed the presence of amide, ester, terpenoid, fatty acid, alkene, terpene, carbohydrate, phenolic and alkane groups. Based on the AB assay, the BGAO- conditioned medium promoted DPSC viability. However, an increase in DPSC cell viability is clearly observed at Day 7 and 14 following exposure in BGAO-conditioned medium at the ratio of 1 mg/mL BG with 50 and 100 µg/mL of AO in comparison with AO alone. BGAO-conditioned medium at a dose of 25 µg/mL supported greater DPSC viability compared to other combination doses. The effect of combination of BG and AO towards DPSC at a certain dosage revealed continuous cell viability over the observation period and promoted cell growth that may be contributed by the combined effects of BG dissolution ions into the culture medium and also the presence of identified compound from the AO extracts namely phytol, linoleic acid, palmitic acid and 1, 4, 7,-Cycloundecatriene, 1, 5, 9, 9-tetramethyl, Z, Z, Z. Thus, it may have a significant potential to help in promoting dental and hard tissue regeneration

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1 INTRODUCTION

The dissolution products of bio-active glass (BG) promote bone cell proliferation and differentiation [3]. The most researched BG is Bioglass® 45S5 which creates strong interface when bonded to bone [4]. BG has been introduced into dental surgery as the simple cones of 45S5 Bioglass® bonded to the bone tissue. It is stable when placed into fresh tooth extraction sites [3] while also promoting interfacial bonding between implantable materials together with the host tissue [5]. BG can be fabricated *via* melt-derived, sol-gel and melt-annealed methods. The sol-gel method has advantages in producing highly porous (micro-porous) BG with higher surface area that demonstrates a greater bioactivity level of the BG and possessed qualities as bone graft materials [6, 7]. This method also enables the production of glasses with high purity and stability [7]. It also reduces the need for high fabrication temperatures. It can be carried out at low temperature and enhances the homogeneity of glasses produced compared to the melt-derived method [8].

Despite that, sol-gel method also has a few drawbacks such as longer time to complete the production process, higher cost of raw materials and shrinkage occurrence during the process of drying and sintering [8]. In the current project, BG powders were prepared through sol-gel processing route since the BG powder produced has several useful ranges of particle size. These are from macrosize to nanosize; the range of 0.5–1.0 mm can be applied for bone repair, while

200–400 µm and 200–600 µm particle sizes are used mainly for periodontal bone regeneration and the 90–500 µm, 500–1000 µm, and 1000–3150 µm particle size ranges for orthopaedic and cranio-maxillo-facial surgery applications [9].

Acmella oleracea (AO) is a plant of the *Asteraceae* family with its multiple traditional uses as a remedy for toothache, treatment of rheumatism, stomatitis, gum infection [10] and throat ache [11]. The extracts of this plant have been reported to have several biological activities such as anti-inflammatory [12], antiproliferative [3], anesthetic and antipyretic [4], analgesics [5], antibacterial and antioxidant [13]. Thus, they have the potential to serve as substitutes to conventional medicines. These potentials make this plant a suitable candidate to be explored further in the present study.

Many studies have explored the potential of AO extracts. This plant is rich in various bioactive constituents and considered to possess diverse pharmacological responses [14]. The chemical analyses of AO extracts revealed the presence of alkamides (alkenes & alkynes and amide derivatives) [14], terpenoids, alkaloids, fatty acids, and a mixture of triterpenes [10]. Other active ingredients found in samples of leaf, stem, and flowers of AO were saponins, tannins, steroids, flavonoids, essential oils, amino acids [15]. A previous extensive phytochemical screening reported that the major pungent constituents isolated from this plant was spilanthol. It was the compound reported to be responsible for reducing toothache problem and could stimulate salivary secretion to relieve dry mouth [10].

Dental pulp stem cells (DPSCs) is a type of mesenchymal stem cells commonly isolated from dental pulp of the permanent human third molars [16]. DPSCs were used in this study because it possesses stem-cell properties and characteristics, including self-renewal capability and multi-lineage differentiation [17]. It is also reported that DPSCs was able to induce angiogenesis and possess potential in tissue engineering and treatment of chronic wounds, stroke and myocardial infarctions [18]. DPSCs can also be obtained from extracted teeth from orthodontic and supernumeraries cases, and commonly harvested in the laboratory within 48 hours post extraction.

Studies regarding the effects of BG which promotes proliferation and regeneration of DPSCs are growing recently [2,19]. These studies highlighted the potential of major roles BG can play in dental restoration and as replacement materials. Currently, not many products are available in the market combining bioactive glass with plant extract. Furthermore, no study has been reported regarding the combination of bioactive glass with AO extracts. Therefore, this preliminary project focuses on the phytochemical screening of active component of AO extracts and its potential in enhancing the viability of DPSCs when doped with sol-gel 45S5 bioactive glass. The expected outcomes from this study were to get useful data on the phytochemicals of AO extracts and their effect on the proliferative activity of DPSCs when introduced to sol-gel bioactive glass doped with AO extracts. These findings may lead to the potential of AO extracts to be used as a paste for dental application related to soft and hard tissue inflammation as well as promoting the dental pulp proliferation and regeneration.

2 MATERIALS AND METHODS

2.1 Extraction of *Acmella oleracea*

The AO extracts were prepared by adopting previously published methods by Bae *et al.* [20] and Franca *et al.* [21] using ethanolic extraction method. The dried leaves of AO (80.99 g) was soaked in ethanol (95%) at a ratio of 1 to 10 weight per volume (1g: 10 mL) and placed on an orbital shaker at room temperature at 250 rpm. The extracts were collected and purified every 3 days for a total of 3 extractions. Then, the combined extracts were concentrated in a rotary evaporator at 50 °C. The dried extract were kept at -20 °C until further analysis. The percentage of extraction yield was calculated based on the formula given below.

Percentage of Extraction Yield=Extraction yield / Dried leaves x 100 (Equation 1).

2.2 GCMS analysis of chemical compounds

AO leaf extract was dissolved in 95% ethanol at concentration of 10 mg/mL and filtered using sterile 0.2 µm Whatman PVDF membrane syringe filter. Chemical compound analysis was performed based on modified method of Anholeto et al. using a GCMS system consisting of an Agilent 6890 gas chromatograph coupled with an Agilent 5973 mass spectrometer [22].

Separation was achieved on a HP-5 MS

capillary column (30 m × 0.25 mm × 0.25 µm). The GC analysis was set at: Injector: 220 °C; split mode: 1:10 ratio; oven temperature: 40 °C to 250 °C (2.5 °C min^{-1}); carrier gas: Helium: 1.0 mL min^{-1}; injection volume: 1 µL. MS conditions were set with ionizing voltage at 71 eV. The ionization source was set at 250 °C. Scanning range was set between 30 amu for low mass and 1000 amu for high mass with a solvent delay of 5.00 minutes. The total ion chromatograms (TICs) and mass spectra were recorded using MSD Chemstation Data Analysis software. Mass spectral identification of unknown volatile organic compounds in AO extract was done by comparing them with the compounds in the NIST library that had match quality above 85.

2.3 Preparation of sol-gel bioactive glass

The sol gel BG were synthesized according to the previous study [19] by mixing the reagents of deionized water, 2N nitric acid, TEOS, TEP, $NaNO_3$ and $Ca(NO_3)_2$. The 45S5 BG composition in weight percentages (wt.%) and mole percentages (mol.%) as shown in the Table 1.The mixture was poured into a sealed container and dried for 2 days in oven at temperature 60 °C and followed with 110 °C for the next two days. Then, it was subjected to sintering process at 600 °C for 1 hour producing sol-gel frits. The resulting frit were milled by using planetary micro mill (Pulverisette, Germany) to obtain a fine powder. The sol-gel BG powder was then subjected to sieving to obtain particle size less than 38 µm prior to use and mixed with AO extracts.

Table 1: The 45s5 Bg Composition In Weight Percentages (Wt. %) And Mole Percentages (Mol. %).

Oxide	Weight percentages (wt. %)	Mole percentages (mol. %)
SiO_2	45.00	46.10
Na_2O	24.50	24.40
CaO	24.50	26.90
P_2O_5	6.00	2.50

2.4 Cell culture

DPSCs were purchased from LONZA, USA. Cells at passage four were used for this assay. The cells were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco USA) supplemented with 10% (v/v) fetal bovine serum (FBS), (Gibco, USA) and 1% (v/v) Antibiotic/Antimycotic (A/A) solution (Gibco, USA). Initially, the cells were seeded inside a T75 cm^2 flasks and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h to allow cell attachment. Once the cells reached 80-90% confluence, they were detached using 0.25% trypsin-EDTA (Gibco, USA). For the assay, cells were seeded at a density of $5x10^3$ cells/cm^2 in a 96-well plates based on a previous study by Mohd Noor et al. [19].

2.5 Preparation of AO- and BGAO-conditioned medium

Four different concentrations, namely 25, 50, 100 and 250 µg/mL for AO extracts and four different concentrations for the combination of BG powders (1 mg/mL) with AO extracts (25, 50, 100, 250 µg/mL) were prepared accordingly. The BG powders and AO extracts were weighed using electronic balance and subsequently mixed with the DMEM cell culture medium, followed by incubation in an incubator shaker for 4 h at 37 °C. Then, the AO and BGAO-conditioned medium were filtered using 0.22 µm syringe filter. The conditioned medium was supplemented with 10% FBS and 1% A/A and incubated in a CO_2 incubator overnight prior to use.

2.6 Cells viability using Alamar Blue (AB) assay

The response of DPSCs upon exposure towards BG-, AO-, and BGAO-conditioned medium were evaluated using Alamar Blue assay (Invitrogen, UK). Briefly, once the cells reached 90% confluence, they were trypsinized, counted, and seeded in a 96-well plates (as explained above). The DPSCs were treated with the BG-conditioned medium (1, 2, 4 mg/mL), AO-conditioned medium (25, 50, 100 and 250 µg/mL) and BGAO-conditioned media (1 mg/mL of BG with 25, 50, 100 and 250 µg/mL of AO) followed by incubation.

At designated time points (Days 1, 4, 7, 14 and 21), the conditioned medium was removed from the well plates and washed with 100 µL of DPBS (Gibco, USA). Then, 150 µL of 10% (v/v) AB in DMEM with no phenol red (Gibco, USA) were added to each well and further incubated for 2 hours. Following the 2 hours incubation period, 100 µL of the reaction product was transferred to a black Costar 96 well plate. The fluorescence of AB was measured using a microplate reader (FluoStar Omega, BMG Labtech, Germany) with

excitation wavelength at 544 nm and emission wavelength at 590 nm.

2.7 Statistical analysis

Data were analyzed using one-way ANOVA with *Scheffe* post-hoc test. The results represent the mean values ± standard deviations (Mean±SD) of two independent experiments with four replicates for each experimental group. Non-treated DPSCs were used as control comparing it with DPSCs exposed to all conditioned medium at each time interval points and differences were considered significant when *p*-values were less than 0.05 ($p<0.05$).

3 RESULTS

3.1 GCMS Analysis

The GCMS chromatogram of AO leaf extract is shown in Figure 1. Based on the results, approximately 47 volatile compounds were detected in AO leaf extracts. Table II shows the overall volatile organic compounds present in AO leaf extracts which include groups of amides, phenols, esters, terpenes, fatty acids, carbohydrates, terpenoids, alkanes and alkenes. Amides (27.01%) was the highest abundant group present in AO leaf extract followed by esters (25.18%), terpenoids (15.48%), fatty acids (7.36%), alkenes (5.57%), terpenes (5.28%), carbohydrates (1.79%), phenolics (1.23%) and alkanes (1.23%) groups. Spilanthol (20.47%) was the major compound present in AO leaf extract followed by phytol (11.55%) and hexadecanoic acid, ethyl ester (6.37%).

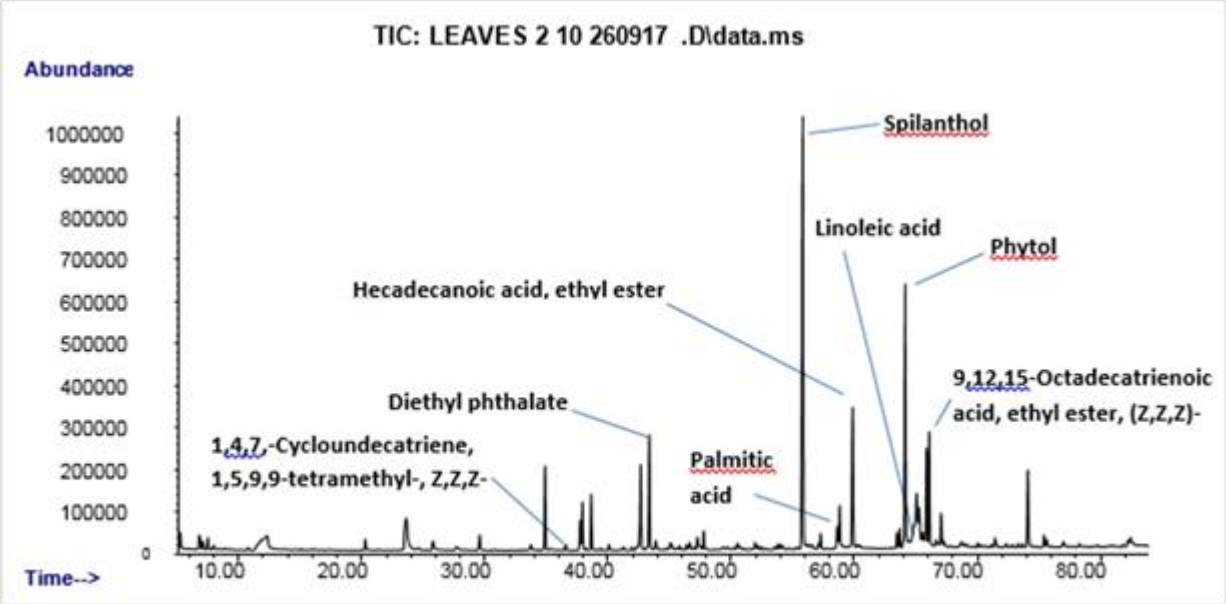

Figure 1: Chromatogram of *Acmella oleracea* leaf extract

Table 2: Gc-Ms Analysis identifying several groups of volatile organic compounds present in *Acmella Oleracea* Leaf Extract. Chemicals are listed in descending order of % abundance

No	RT (min)	Compound Name based on NIST library similarity > 85%	Group	% Area
1	55.857	N-Isobutyl-2(E),6(Z),8(E)-decatrienamide / Spilanthol	Amide	20.47
2	64.184	Phytol	Terpenoid	11.55
3	59.915	Hexadecanoic acid, ethyl ester	Ester	6.37
4	43.407	Diethyl Phthalate	Ester	4.82
5	66.15	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	Ester	4.79
6	65.918	9,12-Octadecadienoic acid, ethyl ester	Ester	4.28
7	65.114	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	Fatty acid	4.23
8	42.71	Caryophyllene oxide	Terpenoid	3.94
9	74.161	N-(2-Phenylethyl)(2E,6Z,8E)-decatrienamide	Amide	3.52
10	34.932	Caryophyllene	Terpene	3.39
11	65.326	N-Isobutylundeca-(2E,4E)-diene-8,10-diynamide	Amide	2.43
12	38.673	1-Pentadecene	Alkene	2.05
13	64.924	9,12-Octadecadienoic acid (Z,Z)- (Linoleic acid)	Fatty acid	2.03
14	37.975	1H-Cyclopenta[1,3]cyclopropa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-methylethyl)-, [3aS-(3a.alpha.,3b.beta.,4.beta.,7.alpha.,7aS*)]-	Terpene	1.89
15	12.337	Glycerin	Carbohydrate	1.79
16	58.858	(E,E,E)-3,7,11,15-Tetramethylhexadeca-1,3,6,10,14-pentaene	Alkene	1.79
17	67.101	Octadecanoic acid, ethyl ester	Ester	1.50
18	37.785	Cyclooctane, 1,2-dimethyl-	Alkana	1.23
19	58.689	n-Hexadecanoic acid (Palmitic acid)	Fatty acid	1.10
20	47.825	2-Propenoic acid, pentadecyl ester	Fatty acid	0.89
21	6.841	Ethylbenzene	Alkene	0.82
22	63.74	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	Ester	0.81
23	29.626	2-Methoxy-4-vinylphenol	Phenol	0.78
24	57.315	Hexadecanoic acid, methyl ester	Ester	0.65
25	63.529	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	Ester	0.63
26	75.471	2-Propenamide, 3-phenyl-N-(2-phenylethyl)-	Amide	0.59
27	20.326	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Phenol	0.46
28	7.116	p-Xylene	Alkene	0.45
29	7.539	1-Butanol, 3-methyl-, acetate	Ester	0.44
30	8.004	o-Xylene	Alkene	0.25
31	36.601	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	Alkene	0.21

3.2 DPSC responses towards sol-gel BG conditioned media

Figure 2 shows the viability of DPSCs upon exposure to sol-gel BG conditioned medium 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 AO extracts. Based on the Alamar Blue assay, the DPSCs viability upon exposure to the BG-conditioned media with 3 different concentrations showed an increase in cell number from Day 1 to 7 in a dose-dependent manner. At earlier time points (Days 1 and 2), the cell viability rate was slower and displayed a similar pattern on all concentrations tested.

It was observed that after Day 2, all cells for each concentration of BG started to show an increase in cell viability for lower BG powder to liquid ratio (1 & 2 mg/mL). However, DPSCs exposed to 4 mg/mL BG powder to liquid ratio showed a decrease in cell viability with the lowest number of viable cells as compared to other concentrations throughout the period of assessment. From the optimization that have been conducted on the effect of 1 & 2 mg/mL of BG combined with different concentration of AO extracts, it was revealed that BG (1 mg/mL) combined with AO extracts showed positive result in promoting DPSC viability. Thus, 1 mg/mL BG powder to liquid ratio combined with AO extracts were selected and further studied.

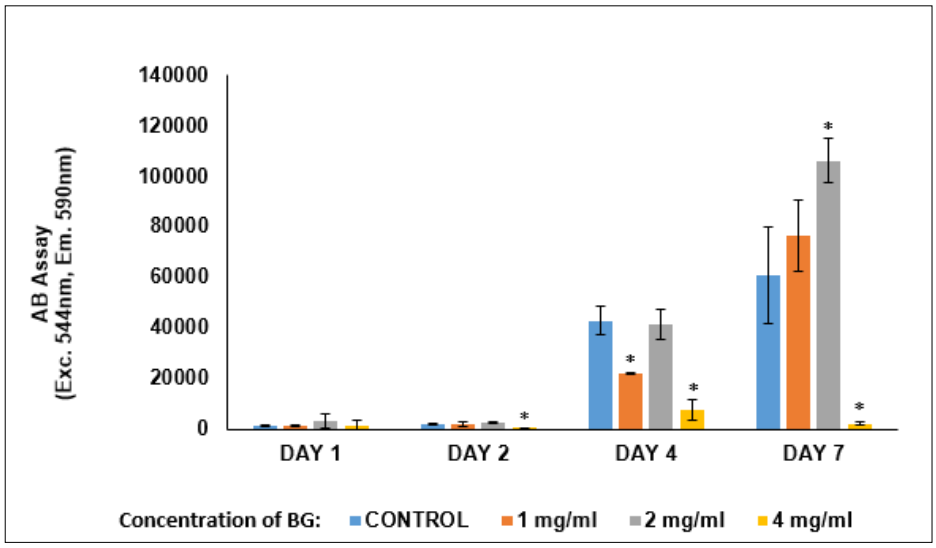

Figure 2: The viability of the DPSC on the BG-conditioned media at Days 1, 2, 4, and 7.

3.3 DPSC responses towards AO extracts

The DPSC responses towards AO extracts were assessed using Alamar Blue assay as shown in Figure 3. This is important to ensure which dose is suitable for DPSC prior to combining the BG with AO extracts. DPSC were exposed to AO-conditioned medium with concentration ranging from 12.5, 25, 50, 100, 200, 250, 500 to1000 µg/mL. Throughout the observation period from Day 1 to Day 2, DPSC showed increasing cell viability when exposed to all concentrations of AO-conditioned medium although there was a decreasing trend observable for all concentrations. As shown in Day 2 & 7, DPSC exposed to AO-conditioned medium with concentration of 25 µg/mL showed the highest cell viability, hence this concentration of AO extract was selected for further analysis and suitable to be combined with sol-gel BG powder.

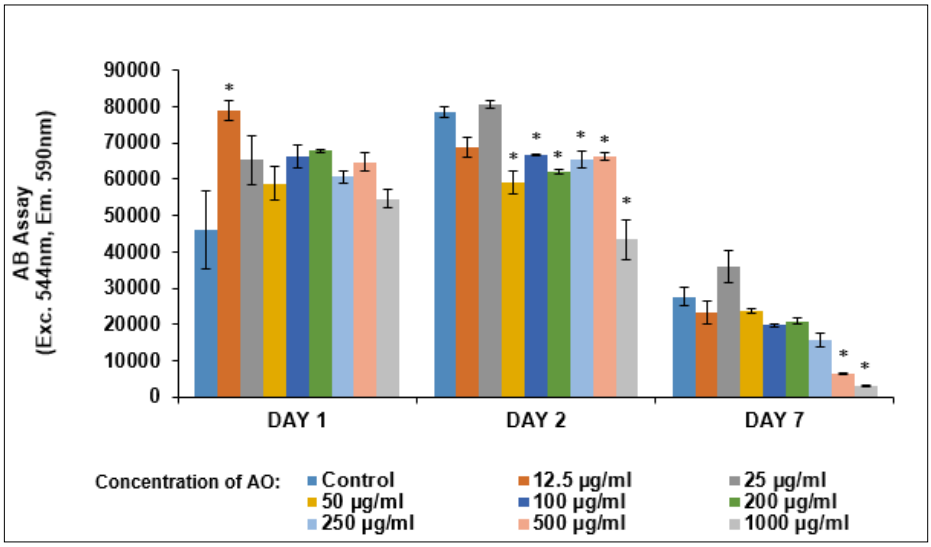

Figure 3: The viability of the DPSC on the AO-conditioned media at Days 1, 2 and 7.

3.4 DPSC responses towards sol-gel BG conditioned media doped with AO extracts

The DPSCs exposed to different doses of AO, BG and combination of BGAO based on Alamar Blue assay is shown in Figure 4. DPSCs exposed to various doses of BGAO revealed continuous increase in cell viability over the observation period from Days 1 to 14 except for the cells treated with 250 µg/mL of BGAO that showed reduction in viability of the cells as shown at Day 7. However, at Day 21, DPSCs showed reduction in cell viability at all concentrations tested which may be due to reduced surface area for cell attachment as the cell number began to increase. BGAO-conditioned medium at a dose of 25 µg/ml supported the highest DPSC viability compared to other doses of BGAO combination that have been carried out in this study. However, in comparison to AO alone-conditioned medium, higher cells viability was observed in BGAO-conditioned medium at the ratio of 1 mg/mL BG with 50 and 100 µg/mL of AO extracts. Results observed at Day 21 showed DPSC exposed to control medium and other concentrations demonstrated lower viability of cells compared to previous days of exposure. The decrease in DPSC viability might be because cells have reached the confluence and may have entered a differentiation phase.

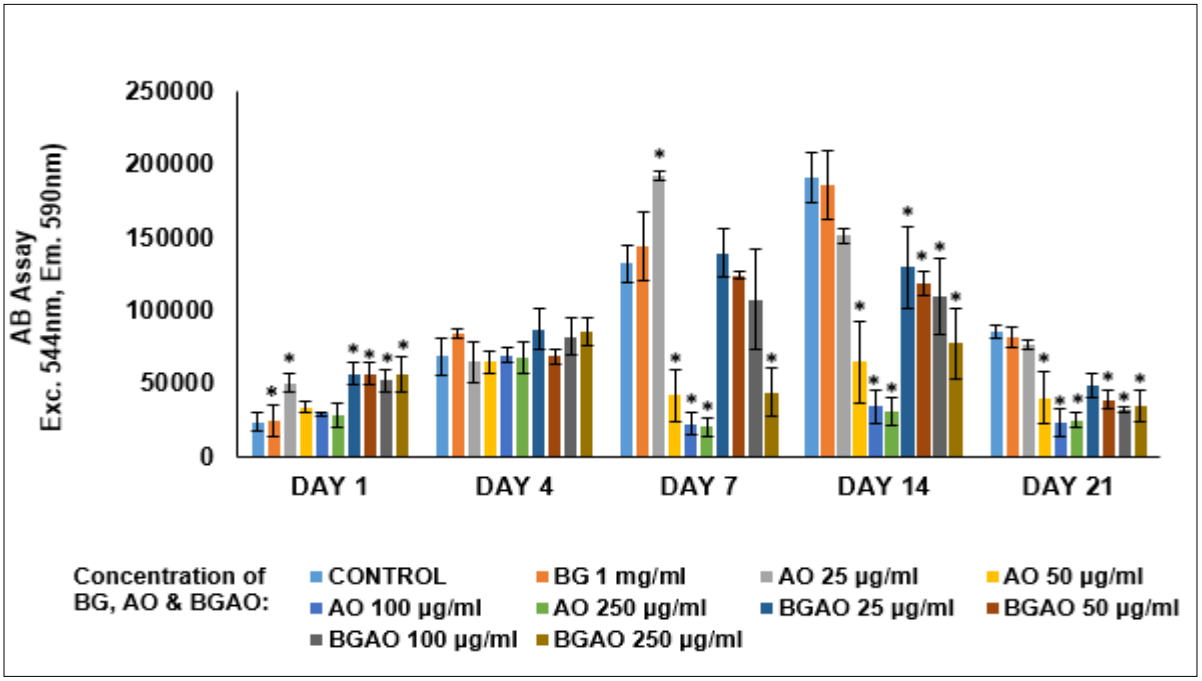

Figure 4: Viability of the DPSCs on the BG-, AO-, and BGAO-conditioned media at Days 1, 4, 7, 14 and 21.

4 DISCUSSION

Spilanthol from the amide group (27.01%) was the most abundant volatile fatty acid detected in the AO leaf extract used in this study. Spilanthol is well known as the most active antiseptic isolated from this plant and also used foranti-inflammatory, antibacterial, antimicrobial conditions [23] as well as for analgesic activity to numb tooth pain [24]. A group of phenols were also found in this plant extracts. From previous study, the presence of phenolic, tannin and flavonoid compounds in the crude ethanol extract of AO leaf is considered as a source for antioxidant activity of this plant [25]. Previous study by Lin et al. (2010) reported that volatile organic compounds from *Eclipta prostrata* extract

which include phytol, linoleic acid, palmitic acid and 1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z, stimulate the proliferation of primary osteoblast cell by increasing the ALP activity [26]. Furthermore, previous study also showed that conjugated linoleic acid promotes osteoblast differentiation in osteoblast cells from mesenchymal stem cells (MSCs) [27]. Therefore, it is suggested that the presence of volatile organic compounds which are phytol, linoleic acid, palmitic acid and 1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl, Z,Z,Z-, in AO leaf extract may contribute to the proliferation of DPSCs observed in the current study.

During the study period, the number of DPSCs seeded into the 96-well plate was kept constant at of $5x10^3$ cells/cm^2, and cells at passage four were used for this assay to ensure that the metabolic activity measured was comparable with one another. AB viability assay revealed that lower amount of powder to liquid ratio of BG (1 to 2 mg/mL) promoted and enhanced the DPSC viability. DPSCs showed lower viability when exposed to higher dose of BG. The result of BG at a dose of 4 mg/mL indicates that they show some toxic effect towards DPSC when compared with lower dose of BG. It was observed that DPSCs exposed to BG-conditioned medium having lower dose showed increase in total number of viable cells throughout the observation period. This suggested that a suitable dose of BG may exist for DPSCs. This result was in concordance with study reported by Houreh et al. on bioactive glass that promotes proliferation and regeneration of DPSCs. This study reported that different compositions of BG-conditioned medium responded differently on stimulation of osteogenic differentiation of DPSCs and effect on cellular behavior [2].

The combination of BG and AO through the effect of BGAO-conditioned medium promoted DPSC viability. It was shown that combination of BG (1 mg/mL) and AO (25 µg/mL) exhibited better cell viability when compared to other doses but was slightly lower than AO alone with the same concentration. However, higher proliferation rate in DPSC viability was observed in BGAO-conditioned medium at the ratio of 1 mg/mL BG with 50, 100 and 250 µg/mL AO in comparison with AO alone. Cell viability was lower at the highest dose of BGAO-conditioned medium compared to the lowest dose. At day 21, the metabolic activity of DPSCs was lower than day 14. 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. It was shown that BGAO is able to promote the viability of cell, which suggests that mixed BG powder and AO extract may be effective for increasing proliferation of DPSCs. A study conducted by Daniele et al. reported that a rhamnogalacturonan, a polysaccharide isolated from AO, promoted epithelial cell proliferation, hence, enhanced tissue regeneration that play important role in gastric healing property or ulcer healing process [28]. AO extracts used in this study also showed similar effect on promoting the proliferation of DPSCs. Lais et al. performed a detailed study on the combination of AO extract with activated charcoal and *Macela* essential oil incorporated in a film in the wound healing test. It showed that the film with the highest concentration of depigmented AO extracts (15.0%) and *Macela* essential oil (1.5%) greatly enhanced its effects on wound repair and cell proliferation when compared with the lower concentration. The result indicated that mixed AO extracts and essential oil improved wound contraction and closure and accelerated the healing activity at specific dose concentration [29]. Based on these and from the data obtained in the present study, it is suggested that the effect of BGAO-conditioned medium on DPSC appeared to follow a similar pattern. Selected concentration of BGAO combination observed in this study promoted viability of DPSC and did not inhibit cell metabolic activity and have great potential in promoting cell growth. However, the results obtained need further clarifications and further work is proposed to observe the binding of BG towards AO leaf extracts. The possible interaction between BG and AO in the chemical level such as whether BG and AO form one complex structure will be determined using NMR analysis in future studies. Further study on finding the possible interaction between BG and AO and other suitable active components within the AO leaf extracts is also crucial. These may help explain the enhanced DPSC response towards the BGAO combination.

4 CONCLUSION

Our finding indicated that the combination of BGAO enhanced viability of human dental pulp stem cells *in vitro*. Thus, it may have a significant potential to help in promoting dental and hard tissue regeneration. In order to increase the benefit from the BGAO combination, further studies on the interactions between BG powder and AO extracts are needed and the mechanism involved warrant further investigation especially at the molecular level.

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