Flaxseed ethanolic crude extract influences growth of stem cells from human exfoliated deciduous teeth (SHED)

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Abstract

Previous study reported that plant natural product promotes stem cell growth. Flaxseed (*Linum usitatissimum*) contains numerous compounds recognized for its health benefits. This study aimed to investigate the biological effects of flaxseed crude extract on stem cells obtained from human exfoliated deciduous teeth (SHED). Whole flaxseeds were ground and extracted with absolute ethanol using soxhlet extractor. The effects of flaxseed crude extract on SHED were assessed at concentration 1, 2, 4, 8 and 16 mg/ml for cell viability using MTT assay, cell morphology using inverted microscope and proliferative activity describe as population doubling time (PDT) using alamarBlue assay. The fatty acid composition of flaxseed was analysed using gas chromatography-mass spectrometry (GCMS) instrumental technique.Although insignificant, flaxseed at concentration up to 4 mg/ml slightly increased SHED proliferation activity while maintaining cell viability and morphology. However, 8 mg/ml of flaxseed inhibited cell viability and proliferation activity, and changed the cell morphology. GCMS analysis revealed the presence of linolenic acid, linoleic acid, palmitic acid and stearic acid. Overall, ethanolic crude extract of flaxseed at concentration up to 4 mg/ml slightly enhanced the growth and maintained the morphology of SHED.

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Introduction

Stem cells from human exfoliated deciduous teeth (SHED) is a type of mesenchymal stem cell with the ability of self-renewal or regenerating and undergo multilineage differentiation.[1]SHED was first identified by Miura in 2003 as immature stem cell population of heterogeneous cells. SHED have become an alternative source in regenerative medicine and dental tissue engineering due to factors such as its high proliferation rates,easy accessibility (obtain from routinely extracted exfoliated deciduous teeth), suitable for long-term storage without affecting its stem cell characteristics and exhibit low risk of immunologic reactions.[2-5] These observations were obtained when stem cells was treated in the presence of common chemical reagents.Interestingly, several studies had reported that natural product from plants could promote stem cell growth.[6-8] Therefore; it would be a huge advantage to identify certain natural product that is non-toxic and can maintain or promote the growth of SHED.

Flaxseed (*Linumusitatissimum* L.) has long been recognized for its health benefits.[9] The traditional and medicinal uses of flaxseed including relief of abdominal pains, constipation, stomach ulcer, skin inflmmation, wounds, and gingival disorder.[10] Flaxseeds are excellent source of omega-3 fatty acid, dietary fiber, unique proteins and phenolic acid. The most

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remarkable compound in flaxseed is omega-3 fatty acid known as alpha linolenic acid (ALA) and phenolic acid known as lignan. Both ALA and lignan compounds are found abundantly in flaxseed than in other plant sources.[11]

More than 50% of flaxseed oil is ALA[12-13] that makes the flaxseed the richest source of ALA. ALA can be metabolized in the body into deocosahexaneioc acid (DHA) and eicasapentaenoic acid (EPA) by the help of certain enzymes.[14] Lignan can be found in most plants but the highest concentration is found in flaxseed. Lignan is a type of phytoestrogen that demonstrate a structure similar to mammalian estrogen (17β - estradiol). In fact, flaxseed was the best precursor of the mammalian lignan.[15] Secoisolariciiesiol diglucoside (SDG) is the major component found inlignanof flaxseed. The SDG is metabolized into lignansenterodiol (END) and enterolactone(ENL) by bacteria in the colon. Previous literatures have reported the antioxidant, anticancer, anti-inflammatory and antidiabetic of SDG.[9,16-17]

To date, more than 90% of the previous studies focused on the utilization of either the oil or lignan extract of flaxseed and only few studies examined on the crude extract which contained both oil and lignan. Also, there is very limited study done looking into the effect of flaxseed on stem cells.Thus, this study aimed to investigate the biological effects of flaxseed crude extract on stem cells obtained from human exfoliated deciduous teeth (SHED). To address this point, cell viability, proliferation activity and morphological observation were assessed on SHED stem.

Materials and methods

Extraction of flaxseed

Flaxseeds (brown type) were obtained from University of Philadelphia, Jordan. Whole flaxseeds were grounded to a fine powder. The weight of ground flaxseed was recorded as initial weight. The extraction was performed using soxhlet in absolute ethanol (99.8%) until the solvent in the upper chamber became clear. The liquid extract was evaporated to remove the solvent using rotary evaporator under reduced pressure at 60 °C. The concentrated extract was then frozen at -80 °C and then freeze-dried for 30 minutes to remove water residue. The weight of crude extract was recorded as final weight. The crude extract was kept in a closed container and stored at 4 °C until further use. The percentage yield of flaxseed extract was calculated using the following equation:

Yield of extract (%) = Final weight (g) / initial weight (g) × 100

Fatty acid screening by gas chromatography-mass spectrometry (GCMS)

GCMS analysis was performed using PerkinElmer Clarus 680/ MS SQ 8 T system equipped with an electron impact ionization source and a 30 m Elite-5ms capillary column. The instrument conditions used for the flaxseed ethanolic extract analysis were as follows: injection volume: 1 µl of sample; carrier gas: helium; flow rate: 1.2 ml/min; split mode ratio: 50:1; oven temperature programme: 40 °C for 1 min, 4 °C/ min to 250 °C, hold for 5 min. The sample was prepared using boron trifluoride method and heat under reflux condenser to minimize the loss of volatile elements during the boiling process: 300 mg of flaxseed extract was dissolved in 7 ml methanolic boron trifluoride (BF_3) solution and heated for 2 min. Then 5 ml of heptane was added and heated for 1 min. The hot solution was allowed to cool to room temperature. Then, saturated sodium chloride was added. The organic phase that was formed at the upper layer was collected. Anhydrous sodium sulphate was added to remove traces of water. The solution was kept in a GCMS vial, ready for injection. The peak of the compound obtained was compared with NIST mass spectra library.

Culture of stem cells from human exfoliated deciduous teeth (SHED)

Cultures of SHED (AllCells, USA) were expanded in alpha-MEM (Gibco, USA) media supplemented with 10% of FBS (Gibco, USA) and 0.5% of Penicillin-Streptomycin (Gibco, USA). The cultures were maintained at 37 °C in a humidified incubator of 5% carbon dioxide (CO_2). The medium was changed every 3 days until the cultures reached 70% confluence. SHED used for the experiments presented here were from passage 15 to 18.

Cell viability by MTT assay and morphological observation

SHED at a density of 1000 cells/well were seeded in 96-well plates and incubated for 24 hours. To observe the effects of flaxseed extract on SHED, 100 µl fresh media containing extracts at a final concentration of 4, 8, 10, 12, 14, 16 mg/ml were added to the wells. For control (untreated SHED), 100 µl fresh media without the extract was added to the wells. After 72 hours of incubation, the morphologies of cells treated at various concentrations of extract were observed using inverted phase contract microscope. The image of cells was captured using Dino-Eye eyepiece camera (Dino-eye, Taiwan). Then, the media in all wells were discarded and rinsed twice with PBS. 50 µl of MTT solution (0.5 mg/ml; (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide; Invitrogen, USA) was added to each well and incubated for 4 hours to allow the formation of blue formazan crystal. The MTT solution was discarded and 200 µl of DMSO (Dimethyl sulfoxide; Nacalai Tesque; Japan) was added to dissolve the blue formazan crystal. The absorbance of the dissolved blue formazan crystal was measured at 570 nm using a microplate ELISA reader (TECAN, USA). The percentage of cell viability was calculated using the following equation:

Cell viability (%) = Absorbance of sample / Absorbance of control × 100

Proliferation activity by AlamarBlue assay

The proliferation activity which is reported as population doubling time (PDT) was assessed using alamarBlue(Invitrogen, USA) assay as described in previous study.[18]Initially, cell density against percentage of reduction was used to plot the standard curve. Cell density of 40 000, 20 000, 10 000, 5000, 2500, 1250, 625, 312.5, 156.35 were seeded in 96-well plates and incubated for 24 hours, followed by the addition of alamarBlue reagent to measure the absorbance. The proliferation activity of SHED treated with flaxseed extract was measured from day-1 to 8. Briefly, 100 µl of medium containing SHED at a density of 1000 cells/well were seeded in 96-well plates and grown to sub-confluent monolayers for 24 hours. Then the medium was discarded, replaced with 100 µl of fresh medium of different concentrations of flaxseed extract (0, 1, 2, 4, 8 mg/ml) and incubated for 24 hours, followed by the addition of alamarBlue reagent to measure the absorbance. Briefly, 10% of alamarBlue reagent was added to the culture medium containing cells and without cells (as background control). After 4 hours of incubation, 100 µl of medium containing alamarBlue reagent was transferred into a new 96-well plate and measured the absorbance at 570 nm, using 600 nm as a reference wavelength .

Statistical analysis

All data were analysed in triplicate and expressed as mean ± standard deviation (n=3). All experiments were repeated at least three times. Data were analyzed using SPSS statistical software, version 17.0 (SPSS Inc, Chicago, IL, U.S.A.). The $p < 0.05$ was considered significant.

Results

Fatty acid compounds of flaxseed by GCMS

The overall yield of crude ethanol extract of flaxseed was 30.23% whereby 83% was in liquid form and 13% was in solid form. Four major peaks were observed in the GCMS chromatogram (Figure 1). The first peak was identified as palmitic acid, followed by linoleic acid, linolenic acid and stearic acid. Based on percentage of peak area, linolenic acid was the highest followed by linoleic acid, palmitic acid and stearic acid (Table 1).

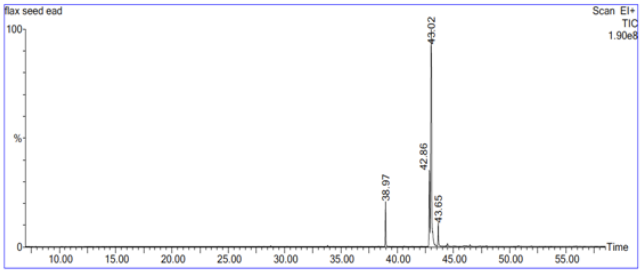

Figure 1. Chromatographic profile of flaxseed crude extract by GC/MS.

Effect of flaxseed extract on cell viability

SHED treated with 4 mg/ml of extract and at lower concentration showed high cell viability comparable to control in MTT, however; it is not significant ($p > 0.05$). In contrast, extract with higher concentration (8 and 16 mg/ml) inhibited

cell viability significantly at 22% and 99% respectively (Table 2).Treatment of SHED with 16 mg/ml of flaxseed crude extract showed a significant cell death after 72 hours of incubation.

Effects of flaxseed extract on cell morphology

The morphology of SHED treated with flaxseed extract of different concentration is shown in Figure 2. SHED maintained its fibroblast-like shape at concentration up to 4 mg/ml of extract. However, the morphology of SHED changed as the concentration of extract increased.

Peak	RT	Fatty acid	Peak under area (%)
1	38.97	Palmitic acid (16:0)	7.48
2	42.86	Linoleic acid (18:2)	12.2
3	43.02	Linolenic acid (18:3)	62.47
4	43.65	Stearic acid (18:0)	4.5

Table 1. Percentage of peak area of fatty acid compounds.

Concentration (mg/ml)	Cell Viability (%)	PDT (hour/doubling)
0	100	42.88 ± 2.43
1	101.15 ± 10.13	43.37 ± 2.79
2	94.05 ± 6.03	41.91 ± 2.45
4	98.45 ± 7.94	40.96 ± 2.70
8	78 ± 12.41*	54.11 ± 8.25
16	0.81 ± 0.24*	No activity

Table 2. Cell Viability and Population doubling time (PDT) of SHED treated with flaxseed extract at different concentration. Values are expressed as means ± SD (n = 3). *Values were significantly different compared to control ($p < 0.05$).

Effects of flaxseed extract on cell proliferation activity

The standard curve of SHED is shown in Figure 3, where greater number of cells resulted in higher percentage of reduction until plateau phase was reached. The proliferation activity of SHED treated with flaxseed extract is shown in Figure 4. All treatment groups (1, 2, 4, 8 mg/ml) showed increased in the percentage of reduction over time. The PDT was calculated based on the percentage of reduction obtained until day-6. The untreated SHED (control) showed a doubling time of 40.45 to 45.31 hours. SHED treated with 1, 2, and 4 mg/ml of extract demonstrated similar PDT with control whereas 8 mg/ml of extract gave 28% higher PDT than control (Table 2). However, the results of the independent t-test revealed that there were no significant differences in PDTs of all treatment groups when compared to the control ($p > 0.05$).

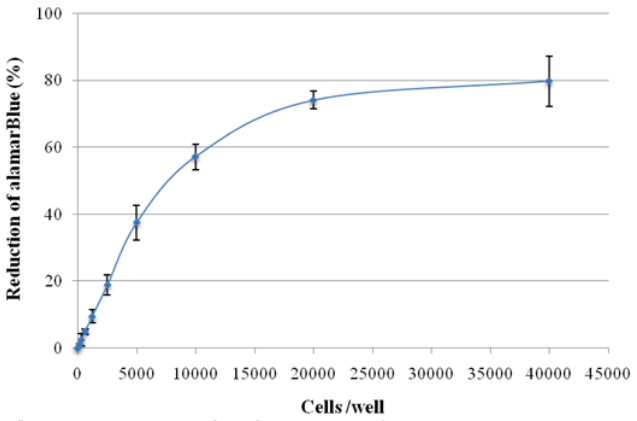

Figure 3. Standard curve of SHED. Data were expressed as means ± SD (n = 3).

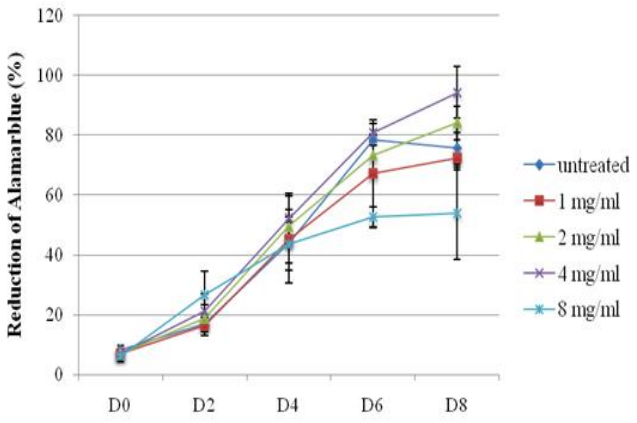

Figure 4. Proliferation activity of SHED treated with flaxseed extract. Data were expressed as means ± SD (n = 3).

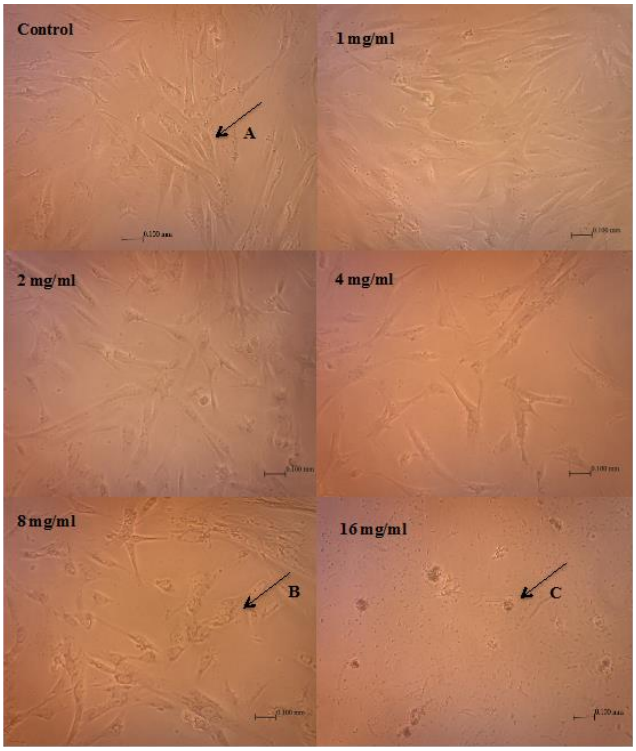

Figure 2. Phase-contrast images (10X magnification) of SHED treated with flaxseed extract at different concentration for 72 hours. The untreated SHED (control) formed elongated and fibroblast-like shape (A). SHED maintained the morphology at concentration 1, 2, 4 mg/ml, however SHED showed cellular swelling, granulation and vacuolation in the cytoplasm at 8mg/ml of extract (B). SHED appeared dark and rounded at 16 mg/ml of extract, indicating dead cells (C).

Discussion

Flaxseeds contain many bioactive compounds that are good for human health. Solvents extraction such as methanol and ethanol are the most common method used for recovering bioactive compounds from plant material.[19-20.] However, the current study only utilized ethanol for extraction due to its less hazardous effect on human and environment.[21] Moreover ethanol extracts are known to exhibit highest antioxidant capacity.[22] Nevertheless, during ethanol extraction, impurities such as free fatty acid, pigments and waxes were co-extracted, which are known to be disadvantageous to oil flavor and stability.[23] The extraction of flaxseed using absolute ethanol gave approximately 25% of liquid or oil and 5% of waxes. The waxy material was considered to be a waste product and was discarded in this study. The oil had darker appearance due to the pigments (chlorophyll and xanthopyll).

Fatty acid (oil) is the main component of the flaxseed extract. The amount of fatty acid in the flaxseed ethanolic extract was in the following order; alpha linolenic acid > linoleic acid >palmitic acid > stearic acid. The order of these fatty acids was consistent with other study.[10] Fatty acids are important component for membrane proliferation in cells as it provides energy storage and precursors of various signaling molecules.[24] Fatty acids are often been added in serum-free media to replace the growth promoting properties in serum.[25] It has been reported that linolenic acid stimulated the proliferation of haemopoietic stem cells instroma-based culture system.[26] In the present study, although it was not statistically significant, it can be assumed that flaxseed increased the proliferation activity of SHED as shown at concentration 2 and 4 mg/ml (Table 2). The linolenic acid in the flaxseed extract may have stimulated the proliferative activity of SHED. In the present study, SHED treated with flaxseed at concentration up to 4 mg/ml showed comparable cell viability (Table 2) and morphology with the control cells (Figure 2). This result suggested that flaxseed is non-toxic to SHED at concentration up to of 4 mg/ml.

It has also been reported that fatty acids inhibited or gave no effect on cell growth.[25]Cell viability and proliferation activity of SHED appeared to be inhibited when treated with higher concentration (8 mg/ml) of flaxseed crude extract. There were also changes in the cell morphology indicated by cell swelling and increased in granulation. Furthermore, none of the cells survived when treated with flaxseed extract at concentration 16 mg/ml. It is suggested that fatty acid at higher concentration incurring strong inhibitory effect on SHED growth. It has been reported that higher concentration of polyunsaturated fatty acids (PUFAs) resulted in apoptosis, and necrosis when the concentrations are even higher.[27] The inhibitory effects of the fatty acid could also related to the carbon chain length and number of double bonds.[28]

Lignan in flaxseed may also have contributed to the inhibitory effects in SHED. Several studies have documented the inhibitory effects and antiproliferative of lignan.[29-30] Lignan inhibit cell proliferation and growth via apoptosis as a result of plant lignan metabolism; enterolactone.[31] The

inhibitory effects in SHED could also be due to the presence of cyanogenic glucoside. İt has been reported that SDG and cyanogenic glucoside presence in high amount in the ethanolic extract of flaxseed.[32] Cyanogenic glucoside is a secondary plant metabolites that are converted to hydrogen cyanide, which are dangerous to human and animal.[33]

Conclusions

To the best of our knowledge, this is the first published report to document the effects of flaxseed ethanolic crude extract on biological activity of SHED. Based on the current study, flaxseed extract is non-toxic to SHED, induces SHED proliferation activity and maintain the cell morphology at concentration up to 4 mg/ml. However, the inhibitory effect of flaxseed extract, which can be partly attributed to both of its oil and lignan components; was demonstrated at the higher concentration. Therefore, the presence of lignan and other phenolic compound in flaxseed ethanolic crude extract should be confirmed in the future study. The concentration of flaxseed that is non-toxic and slightly able to enhance the proliferation activity of SHED is promising especially in tissue regeneration research where it could be used to study the influence of this natural product in differentiation potential of stem cells.

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