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Original article

Date palm fruit (var. Ajwa) promotes proliferation of human bone marrow mesenchymal stem cells: potential natural booster for endogenous stem cells growth

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Summary

Objective – **Maintenance of bone marrow derived mesenchymal stem cells (BMMSC) numbers is important to preserve the balance required for normal tissue regeneration. 'Ajwa' date palm fruit, a popular Middle Eastern food, is known for its superior nutrition and medicinal properties. However, the direct effect of 'Ajwa' dates on stem cell biology remains unclear. This study aims to investigate the potential effect of 'Ajwa' date palm fruit on BMMSC proliferation.** *Materials and methods* – **BMMSC were treated with different concentrations of aqueous extract of 'Ajwa' date palm fruit (mg mL^{-1}). Proliferation, morphology, surface markers and gene expression of BMMSC were evaluated after the treatment.** *Results and discussion* – **'Ajwa' date palm fruit significantly enhanced BMMSC proliferation and retained the stemness of BMMSC. Quantitative gene expression analysis demonstrated significant increase of proliferation related genes; β-Catenin, C-Myc and HGF and lower expression of apoptotic genes; BAX and Caspase-3 and senescence-related gene, p21.** *Conclusion* – **These findings suggest the potential effect of 'Ajwa' date palm fruit as stem cell nutrition in the preservation of endogenous BMMSC for tissue regeneration.**

Keywords

Phoenix dactylifera L., gene expression, nutrition, stem cell growth

Significance of this study

What is already known on this subject?

- Extract of 'Ajwa' date palm fruit contains a variety of nutrients and was proven to have many medicinal effects.

What are the new findings?

- The mechanism behind its general health promoting effect is yet to be discovered. 'Ajwa' date palm fruit was shown to possess some properties as natural booster for endogenous stem cell growth.

What is the expected impact on horticulture?

- The findings could promote date palm fruit consumption as well as the plant cultivation to increase its production. This is due to potential use of date palm fruit as cheaper ingredient in the growth medium for stem cells culture.

Introduction

Date palm fruit (*Phoenix dactylifera* L.) is an ancient crop cultivated mainly in the Middle East. They have been cultivated into distinctive varieties and cultivars, depending on their region of origin, which vary in terms of nutritional value and medicinal properties (Ghnimi *et al.*, 2017). Date palm fruit has been numerously mentioned in Middle Eastern sacred texts (Ahmad *et al.*, 2009). Among the varieties 'Ajwa' date palm fruit is the most revered both for its taste and purported nutraceutical property. It has been specifically mentioned in the book *The Prophetic Medicine* by Ibn Jawziyya to treat various illnesses such as fever, headache, diarrhoea, asthma, inflammation and sleeping disorder (Al-Jawziyya, 1998). Studies have demonstrated the cultivar's superior composition of carbohydrates, vitamins, minerals, and fibres (Al-shahib and Marshall, 2003). Many works also demonstrated its medicinal properties such as anti-oxidant (Vayalil, 2002), neuroprotective (Zangiabadi *et al.*, 2011), nephroprotective (Saafi-Ben Salah *et al.*, 2012), hepatoprotective (Abdelaziz and Ali, 2014), anti-tumor (Rahmani *et al.*, 2014), and anti-bacterial (Taleb *et al.*, 2016). However, the beneficial effect of 'Ajwa' date palm fruit on stem cell biology is yet to be explored.

Regenerative medicine is an emerging technology which concerns cellular regeneration to restore functions of damaged tissues and organs. Mesenchymal stem cells (MSCs) serve as a natural source of undifferentiated multipotent cells that play an important role in the regeneration process of injured tissues. The MSCs were first isolated from the bone marrow (Friedenstein *et al.*, 1968) and eventually they were discovered in various tissues such as periodontal ligaments (Liu *et al.*, 2011), placenta (Pelekanos *et al.*, 2016), and dental pulp (Tsai *et al.*, 2017). They are capable of self-renewal and differentiate into mesodermal cells (Pittenger *et al.*, 1999) including transdifferentiation of different lineages (Li *et al.*, 2010; Sasaki *et al.*, 2008). Moreover, MSCs are capable of stimulating other cells to facilitate tissue repair and regeneration via the paracrine effect (Liu *et al.*, 2017) and immunomodulatory properties (Zhang *et al.*, 2013). It has been

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widely proposed that MSCs migrate to injury site, undergo differentiation and proliferation, promoting tissue repair in pathological conditions such as bone fracture (Alm *et al*., 2010), liver injury (Chen *et al*., 2010), burns (Hu *et al*., 2013), and cardiomyopathy (Marketou *et al*., 2015).

According to data from the U.S. National Library of Medicine, 351 studies used bone marrow derived MSCs (BMMSC) out of 801 MSCs-based clinical trials, as of January, 2018 (National Library of Medicine, 2018). However, clinical trial failures have been frequently reported for MSC therapies (Trounson and McDonald, 2015). In 2016, Squillaro and colleagues revealed the poorly established data regarding the long-term safety of MSCs-based therapy. Based on their analysis in 2015, only 29 studies managed to progress to phases III and IV whereas most studies remained in phases I and II (Squillaro *et al*., 2016). This scenario portrays the challenges in establishing MSCs-based therapies as treatment for diseases. Thus, other alternative approaches utilizing similar benefit and regenerative properties of stem cells need to be explored. One such option is through stimulation of endogenous stem cells with proper nutrition.

As the main concept of stem cell nutrition is to stimulate the release of adult stem cells from bone marrow into the blood stream, the MSC derived from the bone marrow were used in this study instead of other types of stem cells.

Materials and methods

Aqueous extract of 'Ajwa' date palm fruit

100 g of 'Ajwa' date palm fruit (purchased from Saudagar Kurma Sdn Bhd, Malaysia) were weighed, washed, and blended in 1,000 mL of distilled water. The extract was then filtered with gauze followed by a filter paper (Whatman #1) and dispensed into several 50-mL falcon tubes. The O-ring of the tubes were covered with parafilm, pricked few times and stored in -80 °C before freeze-dried. The dry extract was weighed at selected concentration, diluted in DMEM culture medium (Gibco, USA) supplemented with 1% antibiotic antimycotic (AA), 2% FBS (Gibco, USA) or without FBS and filtered using 0.45 µM filter.

Cell proliferation assay

BMMSC were seeded in a 96-well plate with 2,500 cells well^{-1} in 250 µL DMEM, 10% FBS and 1% AA. The plates were placed in humidified incubator with 5% CO_2 at 37 °C for 24 h to allow formation of fibroblast monolayer. After 24 h, the media were replaced with treatment media containing 250 µL DMEM, 1% AA, 2% FBS, or without FBS, and enriched with different concentrations of 'Ajwa' dates; 5, 10, 15, 20, 25 and 30 mg mL^{-1}. The cell viability was read after 24 h, 48 h, and 72 h of treatment using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The media were removed and replaced with 100 µL of DMEM, 1% AA without FBS. 10 µL of MTT reagent were added to each well with final concentration of 0.5 mg mL^{-1} and incubated for 3–4 h. 100 µL of dimethyl sulphoxide (DMSO) were added to dissolve the crystals by shaking for 10 min in the microplate reader (Tecan Infinite M200 Pro). The optical density (OD) was then measured at 570 nm. The tests were carried out in triplicate and repeated three times (n = 3). The percentage of BMMSC viability was calculated based on the cell viability formula (Equation 1) and the result was presented as mean ± standard error of mean (SEM).

$$\text{Cell viability (\%)} = [(A_{sample} - A_{blank})/(A_{control} - A_{blank})] \times 100 \quad (1)$$

BMMSC treatment for the evaluation of cell morphology, surface marker expressions and gene expressions

Morphological observation, quantification of surface marker expressions and gene expressions were performed on BMMSC treated with medium containing 10 mg mL^{-1} of 'Ajwa' dates for 48 hours. The treatment groups were differed in terms of the FBS content. One group was supplemented with FBS (FBS) and the other one without FBS (WFBS). Both treatment groups were compared to control groups without 'Ajwa' dates extract.

Morphology

A direct visual investigation was made under an inverted microscope (100 ×) to observe any morphological changes in BMMSC monolayer culture.

BMMSC surface marker verification

BMMSC surface markers were verified using Human Mesenchymal Stem Cell Verification kit (R&D System) for the following surface antigen: positive markers (CD90, CD73 and CD105) and negative markers cocktail (CD45, CD34, CD11b, CD79A and HLA-DR). After 48 hours of treatment, treatment media were removed and the monolayer cells were washed with phosphate buffered saline (Gibco, Invitrogen). Accutase (Gibco, Invitrogen) was used to detach cells from the wells. Cell suspensions were transferred into falcon tube and centrifuged at 300 × g for 5 min. The supernatant was discarded and the cell pellet resuspended in 200 µL of staining buffer. 100 µL of cell suspensions were transferred into two flow cytometry tubes; unstained and stained. For the stained tube, 10 µL of both each positive and negative marker cocktails were added into the tube and incubated for 30 to 45 min at room temperature (RT) in the dark. Following incubation, the cells were washed with 2 mL of staining buffer to remove any excess antibodies. The final cell pellet and the unstained tube were resuspended in 400 µL of staining buffer for flow cytometric analysis (BD Facs Canto II).

Gene expressions quantification using quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from the cells using TRI Reagent according to the manufacturer's instruction (Molecular Research Center). The complementary DNA (cDNA) was then synthesized using SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Thermo Fisher Scientific). qPCR amplification was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) on the AriaMx Real Time PCR machine (Agilent Technologies). The primer sequences were adapted from articles as listed in Table 1 and made by 1st BASE Company. PCR program was set up as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 1 min. The relative mRNA expressions were calculated using Pfaffl method using TATA box binding protein (TBP) as reference gene.

Statistical analysis

Data obtained from the experiment were compared to control and the results were statistically analysed by SPSS version 20 using non-parametric test, Mann-Whitney U test. Results were expressed as mean ± S.E.M.

Table 1. List of primers for reference gene and target genes. The primer sequences were adopted from published articles.

Genes	Primer sequences (5'-3')		References
TBP	F	TTC GGA GAG TTC TGG GAT TG	(Kang *et al.*, 2015)
	R	GGA TTA TAT TCG GCG TTT CG	
β-*catenin*	F	GCTGATTTGATGGAGTTGGACATGG	(Xiaoyan and Xiangdong, 2013)
	R	GCCAAACGCTGGACATTAGTGG	
C-Myc	F	AATGAAAAGGCCCCCAAGGTAGTTATCC	(Ge *et al.*, 2015)
	R	GTCGTTTCCGCAACAAGTCCTCTTC	
bFGF	F	CCG TTA CCT GGC TAT GAA GG	(Tan *et al.*, 2016)
	R	ACT GCC CAG TTC GTT TCA GT	
VEGF	F	CCCACTGAGGAGTCCAACAT	(Hayati *et al.*, 2011)
	R	AAATGCTTTCTCCGCTCTGA	
HGF	F	CTGGTTCCCCTTCAATAGCA	(Hayati *et al.*, 2011)
	R	CTCCAGGGCTGACATTTGAT	
BCL-2	F	TCCCTCGCTGCACAAATACTC	(Minutolo *et al.*, 2012)
	R	ACGACCCGATGGCCATAGA	
BAX	F	TGGAGCTGCAGAGGATGATTG	(Xu *et al.*, 2008)
	R	GAAGTTGCCGTCAGAAAACATG	
Caspase 3	F	GCA GCA AAC CTC AGG GAA AC	(Zhuo *et al.*, 2009)
	R	TGT CGG CAT ACT GTT TCA GCA	
p16	F	CAACGCACCGAATAGTTACG	(Knösel *et al.*, 2014)
	R	CTGCCCATCATCATGACCTGG	
p21	F	TGGAGACTCTCAGGGTCGAAA	(Al-Haj *et al.*, 2012)
	R	GGCGTTTGGAGTGGTAGAAATC	

Results

Cell proliferation assay

Proliferation of BMMSC were higher in WFBS as compared to FBS. The induction of BMMSC proliferation in WFBS groups were significant for all concentrations of extracts except for 30 mg mL^{-1} after 72 h of treatment. Highest proliferation of BMMSC were seen in 10 mg mL^{-1} to 25 mg mL^{-1} concentrations for both groups. However, treatment with 30 mg mL^{-1} of the extract for 72 h greatly reduced the viability of BMMSC to less than 25% (Figure 1).

Morphology of BMMSC after treatment with 10 mg mL^{-1} date palm fruits extract

Generally, BMMSC in both control and treatment groups exhibited a homogeneous morphology, with spindled and elongated fibroblast-like cells (Figure 2).

BMMSC surface marker

Table 2 depicted positive expressions of MSC-associated surface markers in a range between 80–92%. The statistical analysis revealed no significant difference between treated and control groups.

Genes expression analysis

1. Proliferation-related genes. WFBS medium with extracts significantly induced the expressions of β-Catenin, C-Myc and HGF while only β-Catenin and HGF showed similar pattern of expressions in FBS treatment group. Relative expression ratio of β-Catenin in FBS group (1.36) showed higher and significant expression compared to WFBS ($P < 0.05$). HGF was significantly expressed in both groups, WFBS (1.05) and FBS (1.16). However, VEGF and bFGF expressions were significantly reduced in treatment group of WFBS. In FBS group, only bFGF showed significant reduction.

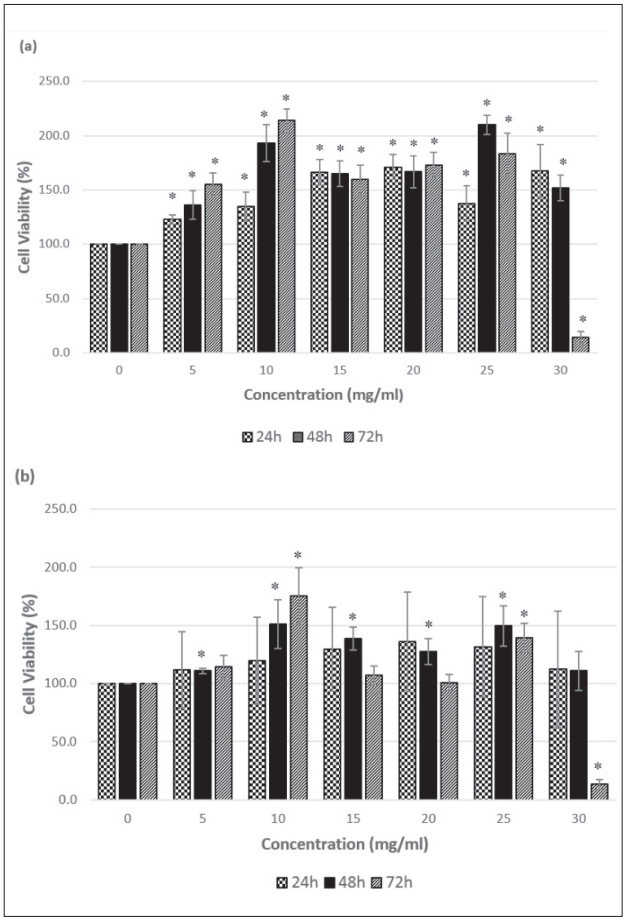

Figure 1. The proliferative effect of 'Ajwa' dates extract on BMMSC (a) without FBS, and (b) with 2% FBS. *indicates significant difference at $p < 0.05$ using Mann-Whitney U test.

2. Apoptosis-related genes. The expressions of BAX and Caspase 3 were lower compared to control. Significant increase of BCL-2 is noted only in FBS group.

3. Senescence-related genes. Relative expression of p21 was significantly lower in both WFBS and FBS groups as compared to control. On the contrary, p16 were increased in both groups with significant induction was shown in the FBS group.

Discussion

Prophetic foods promote BMMSC proliferation

In this study, the proliferative effect of 'Ajwa' date palm fruit extract was observed whereby the percentage of BMMSC viabilities was significantly increased. This was supported by previous findings on mouse spermatogonial stem cells (Mahaldashtian *et al*., 2016) and cardiomyoblastic cells (Al-Yahya *et al*., 2016) which concluded that flavonoid, carotenoid, and estrogenic compounds in 'Ajwa' dates prevented depletion of endogenous antioxidant, hence protecting the cells from free radicals and increased cells proliferation. Recently, in rodent myocardial infarction models, 'Ajwa' dates showed promising effect in mobilizing the progenitor cells from the bone marrow and peripheral circulation to injury site (Alhaider *et al*., 2017). Hence, the concept of stimulating the endogenous stem cells and enhance tissue repair following tissue injury is clinically relevant.

A drastic reduction of cell proliferation was seen with 30 mg mL^{-1} of extract for both groups after 72 h treatment. One of the explanations that could have contributed to this effect is due to high glucose content in 'Ajwa' dates (Assirey, 2015). According to Halliwell (2014), cells exposed to high glucose content for prolonged periods will increase the production of mitochondrial superoxide anion radical (one of the reactive oxygen species (ROS)) and cause glycation and glycoxidation of proteins (Halliwell, 2014). Under certain circumstances,

Table 2. Surface marker expression of BMMSC. Data are presented as the mean ± SEM.

Group	Antigen		
	CD73	CD90	CD105
Control 10% FBS	87.4 ± 0.81	91.6 ± 1.7	82.4 ± 0.2
Control No FBS	81.5 ± 0.09	88.3 ± 0.9	84.1 ± 1.5
No FBS Ajwa	83.2 ± 0.84	90.0 ± 1.0	90.4 ± 1.0
Control 2% FBS	83.3 ± 0.69	87.4 ± 0.1	85.6 ± 0.8
2% FBS Ajwa	82.7 ± 0.18	89.9 ± 1.2	86.1 ± 1.2

Mann-Whitney U test. No significant difference at $p > 0.05$.

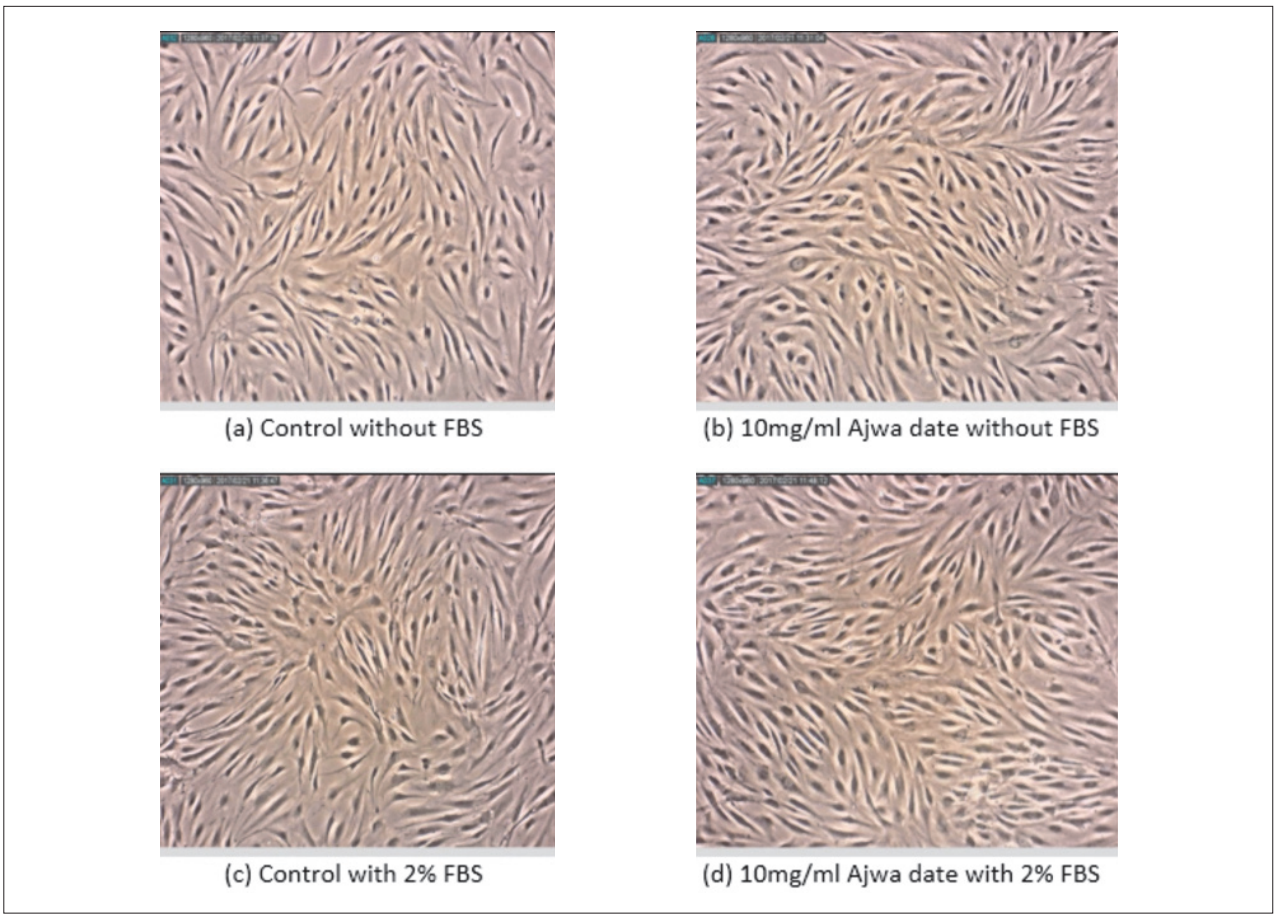

Figure 2. Morphology of control and treatment groups after 48 h treatment (100 ×).

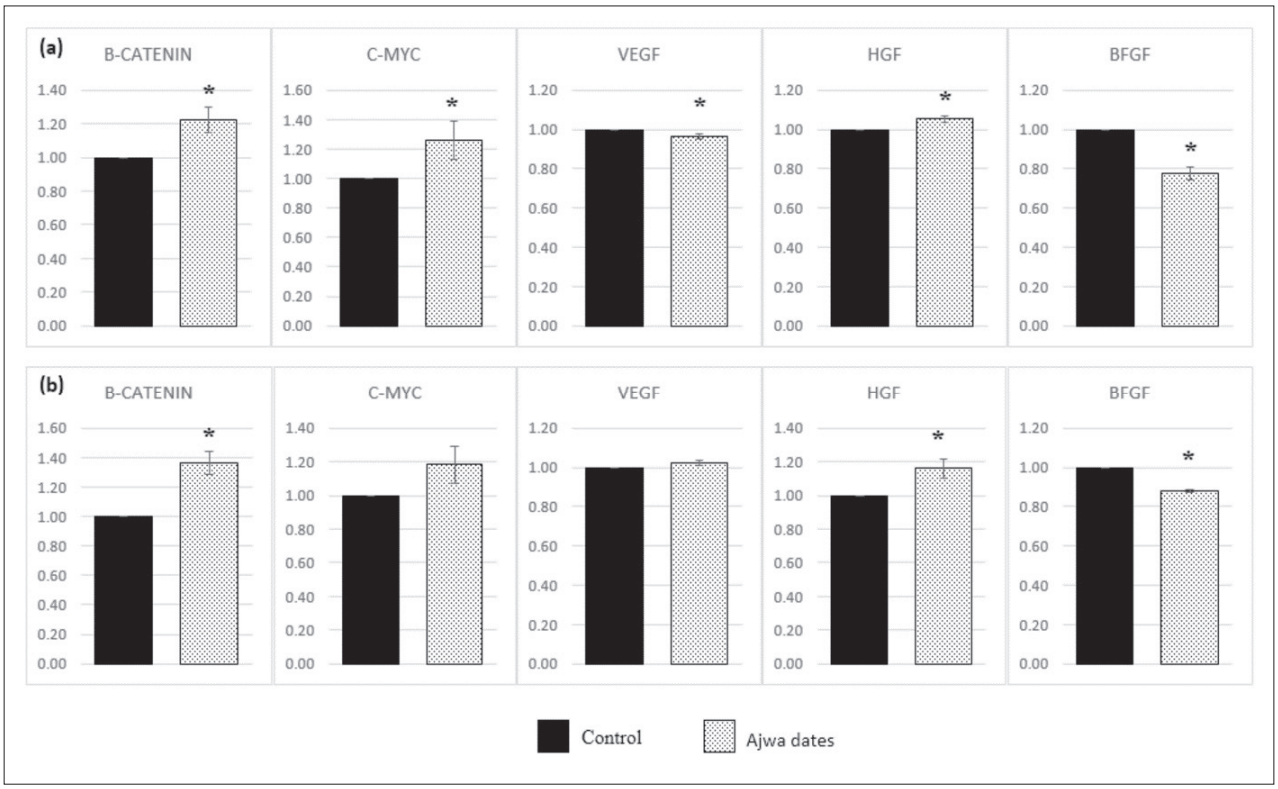

Figure 3. Relative expression ratio (R) of proliferation-related genes after treatment with 10 mg L^{-1} extracts in media (a) without FBS (WFBS), and (b) with 2% FBS (FBS). *indicates significant difference at $p < 0.05$ using Mann-Whitney U test.

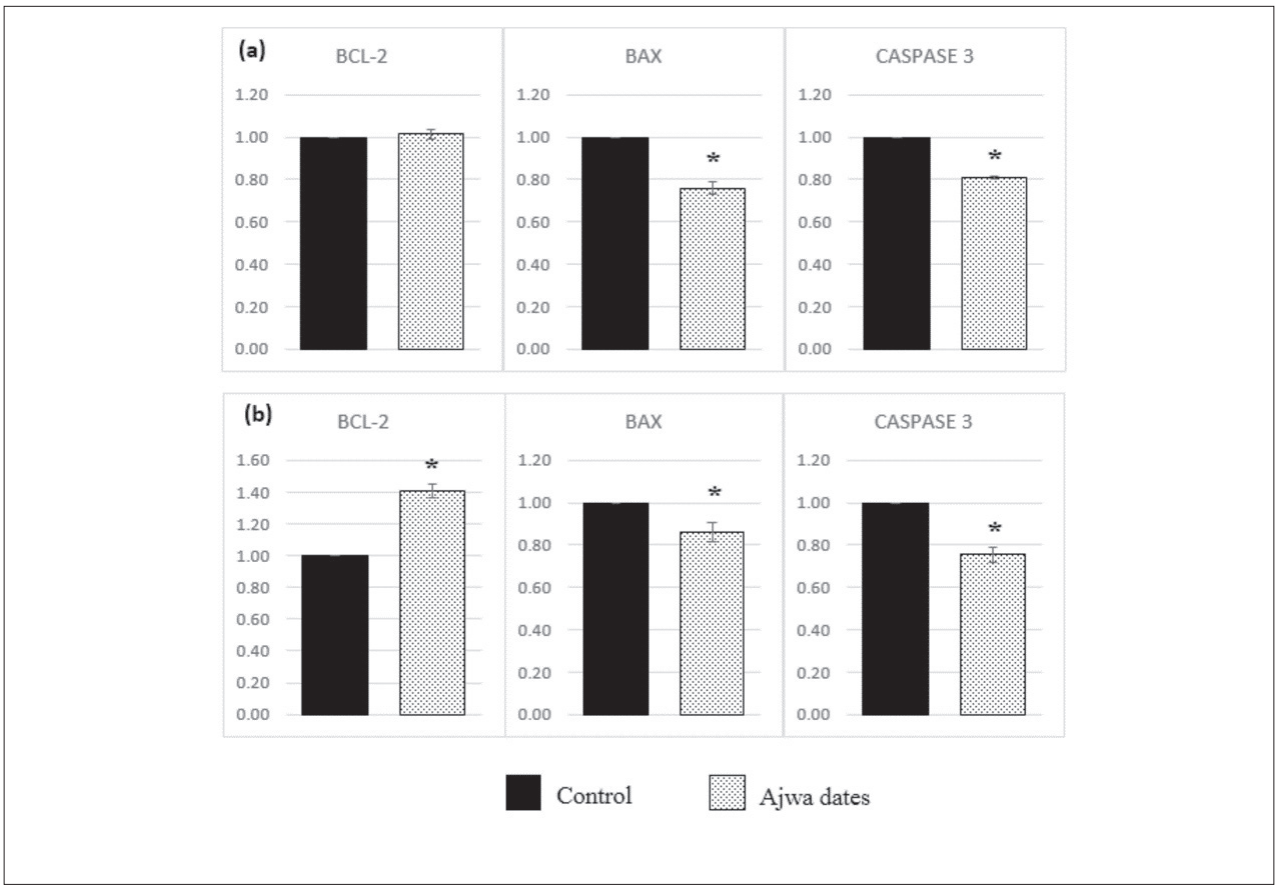

Figure 4. Relative expression ratio (R) of apoptosis-related genes after treatment with 10 mg mL^{-1} extracts in media (a) without FBS (WFBS), and (b) with 2% FBS (FBS). *indicates significant difference at $p < 0.05$ using Mann-Whitney U test.

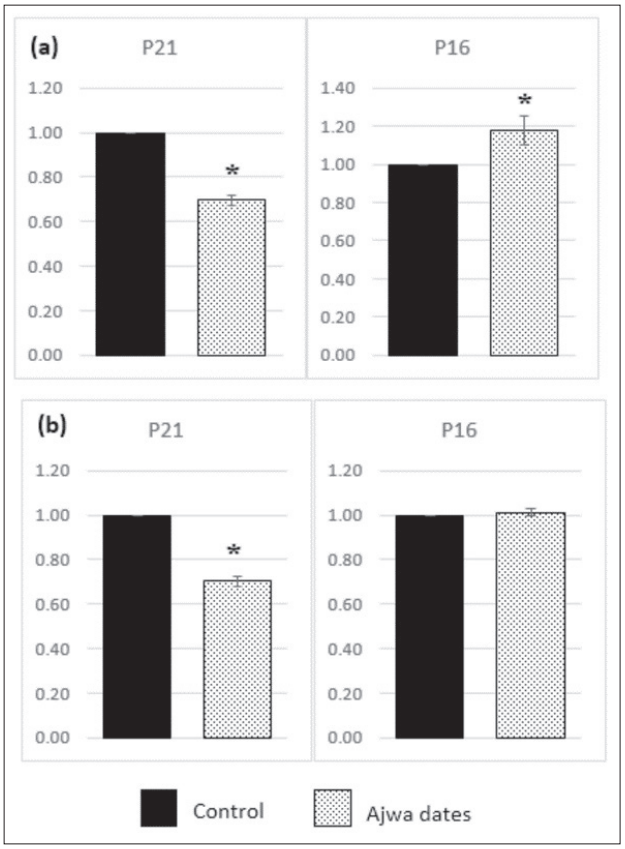

Figure 5. Relative expression ratio (R) of senescence-related genes after treatment with 10 mg mL^{-1} extracts in media (a) without FBS (WFBS), and (b) with 2% FBS (FBS). *indicates significant difference at $p<0.05$ using Mann-Whitney U test.

serious imbalance between the level of ROS and antioxidant defences will disturb the cells metabolism and damage the cells (Lushchak, 2014). This phenomenon is known as oxidative stress. It impairs the stem cell functions, induces apoptosis and cells senescence (Li *et al*., 2007; Stolzing *et al*., 2006). Another factor that may inhibit BMMSC proliferation is the effect of HGF increased expression. Forte and co-workers reported that short-term exposure of mouse MSC to HGF increased its proliferation while long-term exposure resulted in decreased cell proliferation (Forte *et al*., 2006).

Prophetic foods retain BMMSC stemness

Morphology of control and treated BMMSC illustrates similar features of MSC seen in previous study (Haasters *et al*., 2009). In other words, this could possibly mean that BMMSC did not differentiate into different cell types and retains its stemness state. This morphological-based assumption was further supported by the high expression of positive markers CD73, CD105 and CD90 and the lack in expression of other markers including CD45, CD34, CD14, or CD11b, CD79 alpha or CD19 and HLA-DR surface molecules after the treatment of BMMSc with the extracts (Dominici *et al*., 2006). No significant reduction of MSC surface markers may suggest minimal changes in BMMSC phenotypes.

Gene expression

β-Catenin is a key molecule that is involved in a canonical Wnt signalling pathway. This pathway is crucial in regulating the self-renewal and differentiation of BMMSC (Zhu *et al*., 2014). Accumulation of unphosphorylated β-Catenin in the cytoplasm causes nuclear influx and transcription of target genes such as C-Myc (Ling *et al*., 2009). The present study demonstrated a significant increase of β-Catenin and C-Myc expressions in both groups. However, treatment with date palm extracts and 2% FBS co-stimulate the β-Catenin expression of BMMSC better than treatment WFBS. Minimal concentration of FBS was used to give minimal supports to the cells as FBS was long known as vital nutrient essential for MSC culture which provides growth and adhesion factors to the cells (Díez *et al*., 2015). A relationship between β-Catenin and C-Myc expression could be seen from our result. The expression of C-Myc which is directly proportional to the expression of β-Catenin confirmed previous findings (Li *et al*., 2012; Zhang *et al*., 2012).

Apart from Wnt/β-Catenin, there are other growth factor signaling pathways involved in MSC proliferation and in addition the growth factors mostly produce/results into multiple biological effects. Other than proliferation, they may also cause changes in cells motility and survival (Rodrigues *et al*., 2010). Different pattern of growth factors expressions was detected in this present study. Most notably, HGF expression was significantly increased in both 'Ajwa' dates treatments supporting the proliferative effect of the extract. On the other hand, the VEGF and bFGF expressions were generally reduced after treatment with the extract. Both growth factors are potent angiogenesis stimulators and were proven to significantly increase in angiogenesis model (Fariha *et al*., 2013). However, it was shown that bFGF expression was greatly stimulated in a culture condition of lower cell density (Tsutsumi *et al*., 2001). As bFGF was only measured after 48 h of treatment, the increased of bFGF might not optimal at this time of point and we speculate it has just reduced after maximum increase at 24 h of treatment.

A reduced expression of apoptotic genes; BAX and Caspase 3 in both groups suggest low incidence of BMMSC apoptosis. Meanwhile, expression of BCL-2 was slightly increased compared to control which could be due to its role as pro-survival protein (Czabotar *et al*., 2013). This finding also confirmed a previous finding which state that expressions of BAX and BCL-2 were inversely proportional (Paul-Samojedny *et al*., 2005).

Additionally, the present study suggests low incidence of cells undergoing senescence as BMMSC expressed low p21 in both groups with different p16 expression. Recently, involvement of Wnt/β-Catenin signaling has been proposed in cell senescence of human mammary artery cells (Marchand *et al*., 2011) and MSC (Zhang *et al*., 2011). These studies found that Wnt/β-Catenin signaling was elevated in aged tissue but whether the cell senescence pathway is through p16 or p53/p21, remains unclear. From our result, a similar pattern of relative expression was seen in p16 and β-Catenin. Hence, Wnt/β-Catenin could possibly induce the BMMSC senescence through p16 pathway.

Conclusion

'Ajwa' date palm fruit successfully induced the proliferation of BMMSC as compared to control. The proliferative effect of 'Ajwa' date palm fruit could be possibly induced through Wnt/β-Catenin and HGF signalling. Furthermore, the treatments did not alter BMMSC stemness but reduced apoptosis of BMMSC. Lastly, a reduced expression of senescence gene, p21 suggests a low incidence of cells undergoing senescence through p53 pathway. However, an increased in p16 expression could be related to the Wnt/β-Catenin sig-

nalling. Further tests are needed to verify the potential effect of 'Ajwa' date palm fruit as an ideal nutrition for stem cell growth and stimulation of their mobilisation to damaged tissues.

Acknowledgments

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Conflict of interest

There is no conflict of interest.

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