Effect of aqueous extract of ajwa dates on C2C12 myoblast cell proliferation, migration and differentiation

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Abstract. Chemothepapeutic agents generate side effects or cytotoxicity to other cells that include muscle cells. Doxorubicin, one of the mostly used chemotherapeutic agents, has been known to cause a loss of skeletal muscle mass which known as cachexia. On the other hand, chemopreventive agents, which mostly derived from phytochemicals, have been known to show less cytotoxicity to cancer cells than chemotherapeutic agents. However, their effects on the muscle cells remain largely unknown. In this study, we investigated the effect of ajwa dates extract in C2C12 myoblast cells. C2C12 cells are satellite cells or adult muscle stem cells which can differentiate to from multinucleated myotube. MTT assays had been performed to determine the effect of ajwa dates extract on cell viability. In addition, effects on cell migration were examined by wound healing method. Proliferation assay was done to investigate the effect of ajwa dates extract on cell growth. Furthermore, C2C12 differentiation into myotubes was also investigated. The results of the cell viability assay against C2C12 cells showed that low concentration of ajwa dates was not toxic for C2C12 cells, whereas higher concentration of ajwa dates (10 µg/µl) decreased cell viability. In addition, the effect of ajwa dates extract (1 µg/µl) on cell migration depended on cell seeding density. At a lower cell density, the extract might promote myoblasts migration, whereas at a higher cell density it inhibited the cell migration that may be related to the induction of myocytes differentiation. Overall, we found that ajwa dates extract exhibited low or no cytotoxicity on C2C12 myoblast cells.

1. Introduction

High demand of energy in cancer cells can result in the abnormalities of energy metabolism of normal cells of the cancer patients, these abnormalities may also contribute to the cancer related deaths [1]. Since the increase of energy expenditure in cancer cells is not supported by the energy intake from nutrients consumed by the patients, to fulfill the energy requirements, the cancer cells secrete cytokines such as TNF-α and IFNγ that induce muscle wasting and fat degradation, the condition known as cancer cachexia [2]. On the other hand, cancer cachexia is also promoted by chemothepapeutic agents such as doxorubicin, anthracycline, cyclophosphamide, 5-fluorouracil (5-FU), cisplatin, or methotrexate [3][4][5]. To reduce the risks of patient morbidity, studies to find the strategies of cancer treatment along with the inhibition of cancer cachexia are important to be performed.

Ajwa date (Phoenix dactylifera L.) is one type of dates or palm fruit Ajwa dates contain polyphenols which show inhibitory effects on Caco-2 colon cancer cell growth. In addition, other pharmacological activities of ajwa dates include antioxidant, antiinflammator, antimicrobial, nephroprotective as well as hepatoprotector [6]. Dates extract also exhibits inhibitory effects in several cancer cell such as prostate cancer cells (P3C), human breast adenocarcinoma (MCF7), and human...
hepatocellular carcinoma (HepG2) [7] [8] [9]. In addition, combination of ajwa date and chemotherapeutic agents can improve the treatment outcome by reducing microbial infection [10]. In this study, we investigated the effect of ajwa dates extract in C2C12 myoblast cells to study the safety of dates extract to be developed as an adjuvant for cancer that can decrease cancer cachexia.

2. Material and Methods

2.1. Preparation of Ajwa Dates Extract

Ajwa dates were purchased from Indonesian local shop. The preparation method of ajwa dates extract was adapted and modified from Al-Farsi et al. (2003) [8]. Briefly, the dates were dried at 4°C for 24 hours and cut into 0.5-1 cm thick slices. One gram of dates were mixed and stirred with 30 mL of aquadest at 60°C for 2 hours and then filter sterilized. The dates water extract was stored at -20°C in aliquots.

2.2. Cell Culture and Reagents

C2C12 cells were grown in DMEM (Dulbecco's modified Eagle's medium) (Sigma) supplemented with 15% (v/v) heat-inactivated FBS (Fetal Bovine Serum) (Sigma), and 1% (v/v) penicillin-streptomycin antibiotics (Gibco). The cells were grown in a 5% CO₂ incubator at 37°C.

2.3. Cell Viability Assay.

Cell viability assay was performed by the MTT assay. C2C12 myoblast cells were seeded for 24 hours in 96 well plate. C2C12 cells were seeded at a density 8,000 cells/well. The following day, the cells were treated with several concentrations of ajwa dates extract (10; 5; 1; 0.5; 0.25; 0.125 µg/µl) or control solvent (water) for 24 hours. Afterward, the medium was discarded and replaced with 100 µl MTT solution (10% (v/v), 90% (v/v) medium culture) was added and incubated for 2-3 hours. The MTT conversion into formazan crystal was stopped by the addition of SDS solution (10% SDS in HCl 0.01 M). Then, the plate was wrapped by using aluminium foil and stored overnight. The cell viability was analyzed by the measurement of the absorbance at 570 nm.

2.4. Migration Assay.

C2C12 cells were seeded at density 15,000 or 10,000 cells/well in 96 well plate and incubated overnight. The pipette tips were used to created cells free gap. Then, the medium containing ajwa dates extract (1 µg/µl) or water solvent were added. The culture plate was incubated in 5% CO₂ incubator at 37°C. The wound area was measured by taking the picture at 0 and 6 hour incubation time using Nikon Eclipse Ti-S inverted microscope and Nikon DS-Ri2 camera, and analyzed by using Image J to calculated wound closure.

2.5. Proliferation Assay.

C2C12 cells were seeded at a density 8,000 cells/well in 96 well plate for one day. After seeding, cells were treated using 1 µg/µl of ajwa dates extract. The cell viability was analyzed using MTT method after 0, 6, 24, 30 or 48 hour incubation.

2.6. Differentiation Assay

C2C12 cells were seeded 100,000 cells/well in 6-well plate using DMEM medium supplemented with 15% FBS. After 2 days, the medium was replaced by differentiation medium (DMEM supplemented with 2% horse serum and 1% penicillin-streptomycin, containing 1 µg/µl ajwa dates extract or control solvent) and incubated in 5% CO₂ incubator at 37°C. The differentiation medium was periodically changed every 2 days. The myotube formation was observed after 6 day incubation.
3. Results and Discussions

3.1. Cell Viability Assay. 
The effects of ajwa date extract to the cell proliferation of myoblast C2C12 cells were demonstrated by MTT assay and the results were shown on figure 1. The Ajwa dates extract reduced cell viability at concentration 10 µg/µl, while at concentration 0.125 µg/µl exhibited 105 % cell viability. In generally, the result showed that ajwa dates extract was not toxic for C2C12 cells.

3.2. Migration Assay
Ajwa dates extract effect on migration rate of C2C12 cells migration was investigated using wound healing assay. Rate of migration was studied at cells density 10,000 cells/well or 15,000 cells/well of 96-well plate. The result showed that Ajwa dates extract effect on migration rate of C2C12 cells depended on the cell density. At 10,000 cells/well, dates extract significantly induced the rate of cells migration compare with solvent control (P<0.01), while at cell density 15,000 cells/well, ajwa dates extract reduced the rate of the cell migration (P<0.05) (Figure 2).

3.3. Proliferation Assay
C2C12 cells that treated with 1µg/µl Ajwa dates extract showed faster growth rate compared with the cells treated with control solvent or control cell. Based on the results, we hypothesize that ajwa dates extract may affect the C2C12 myoblast cell growth(Figure 3).

3.4. Differentiation Assay
Differentiation assay of C2C12 myoblast cells into myotubes were investigated to observe the effect of dates extract on myoblast differentiation. The results indicated that ajwa dates extract 0.5 µg/µl increased the number of myotubes compared with control solvent. However, the number of differentiated cells was in opposite to the higher concentration of ajwa dates extract. Higher concentration of ajwa dates (5µg/µl) exhibited lower number of myotubes (Figure 4) than lower concentration of the extract (0.5 and 1 µg/µl).

Figure 1. C2C12 cell viability analysis by using MTT assay
Figure 2. Wound healing assay in C2C12 cells. Representative images of (A) wound assay on 100,000 cells/ml of C2C12 cells and (B) wound healing assay on 150,000 cells/ml of C2C12. (C) the percentage wound closure on 100,000 cells/ml (P<0.01) and 150,000 cells/ml (P<0.05) after treated with Ajwa dates extract or control solvent.
Figure 3. Proliferation assay of ajwa dates extract (A) and control solvent (B). C2C12 cell relative growth was investigated by using MTT assay at incubation time: 0, 6, 24, 30 and 48 hours.
Figure 4. C2C12 differentiation assay. The cells were treated with control solvent or ajwa dates extract (A). Number of differentiation cells after treated with various concentration of ajwa dates extract or control solvent (B).

Many studies have been reported the benefit of ajwa dates extract for human life. Digested ajwa dates extract had been investigated by in vitro and in vivo assays as as anti-inflammatory, antioxidant, antidiabetic as well as anticancer [11][12][13][14][15]. Methanolic extract of Ajwa dates was reported to show inhibitory effects on various malignant human cells that include prostate (DU-145 and LNCaP), breast (MCF-7), lung (NCI-H460), gastric (AGS), and colon cancer cells (HCT-116) [11][14]. In addition aqueous extract of Ajwa dates also showed inhibitory activity in hepatocellular carcinoma (HCC) and colon cancer cells (Caco-2) [13].

Ajwa date is a high-energy food that contains glucose and fructose as main contents. The amount of glucose contentis about 20-50% [8]. Ajwa dates are also enhirched with amino acid which include glutathione, spartic acid, proline, glycine, lysine, that cannot be synthesized by human body [8][16]. In addition, ajwa dates also contain dietary fibers, lipid, polyphenols and flavonoids [8]. The nutriens which make up the ajwa dates may be potential for the health of skeletal muscle tissues.

C2C12 cells are adult muscle stem cells or myoblasts which can differentiate into multinucleated myotubes. In this study, the effects of ajwa dates on cell viability, proliferation, migration and cell differentiation were investigated. The result showed that Ajwa dates extract showed low or no toxicity for the cells. Result of migration assay showed that Ajwa dates extract significantly induced the migration rate at cell seeding density 100,000 cells/ml (p<0.001), but the migration rate significantly reducedat cell seeding density 150,000 cells/ml. The percentage of cells growth were analized by proliferation assay in different incubation time. The result showed that cells treated with ajwa dates extract possesses higher percentage of cell growth than the cells treated with control solvent untreated cells. Moreover, the results of C2C12 cell differentiation assay indicated that ajwa dates extract effect on cell differentiation depended on concentration of ajwa date extract. Lower concentration of the extract was found to promote myotube formation.

Variety of the nutrients that comprise in ajwa dates may affect the proliferation, migration and differentiation of murine C2C12 myoblast cells. Sugar as the main con of the ajwa dates may play a role towards C2C12 energy metabolism. It has been reported that to start the differentiation stage, C2C12 cells need approximately 60% of their energy that originated from glucose. However, the higher concentration of glucose results in negative effect on cell proliferation and differentiation since the higher concentration of glucose can increase basal cellular and mitochondrial respiration which generate stress condition [17].

4. Conclusions

Ajwa dates extract showed low or no cytotoxic effect in C2C12 myoblast cells. In addition, the effect of ajwa dates extract oncell migration depends on cell seeding density. At lower cell density, the extract
might promote myoblasts migration, whereas at higher cell density it might enhance myocytes differentiation thus inhibiting cell migration. Further studies are required to study the combination effect of ajwa date extract with chemotherapeutic agents on the metabolism of C2C12 cells which represents muscle wasting in cachexia.

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6. References
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