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EFFECTS OF DATE SEED EXTRACTION (ROASTED AND NON-ROASTED) AS A POTENTIAL TO INHIBIT ADIPOGENESIS OF BONE MARROW MSCS

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Abstract

Introduction: Mesenchymal stem cell (MSCs) are multipotent stem cells and can differentiate into different cell types and can proliferate for long time. These two characteristics make MSCs an excellent source for regenerative medicine to treat various diseases and injuries.

Objectives: The objectives of the present study are to find the effects of date seed extraction (roasted and non-roasted) as a potential to inhibit adipogenesis of bone marrow MSCs.

Materials and Methods: Bone marrow mesenchymal stem cell lines (human MSCS) is a gift from our collaborator from Denmark Professor Mousta Kassems lab in Odense. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) that includes D-glucose 4500 mg/L, 110 mg/L sodium pyruvate, 4 mM L-glutamine, 10% fetal bovine serum (FBS), non-essential amino acids (Gibco-Invitrogen, USA), and 1x penicillin–streptomycin (Pen–strep).

Results: The viability of the cells grown has been evaluated using the AlmarBlue according to the standard protocol described. A total of six plates were divided between roasted and non-roasted date seed extracts. The cell viability of these plates was measured on day 1, day 2 and day 3 using AlmarBlue. Moreover, we measured the cell viability of the remaining two plates of the 24 well-plate on day 9 for cells incubated with different concentrations of roasted and non-roasted date seed extract with or without adipocyte induction medium CNT. The intensity of fluorescence is proportional to the number of living cells in treated samples and CNT. AlmarBlue assay showed that roasted date extract manifested high cell viability on day 1 and day 2 compared to day 3 which showed a reduction in cell viability. The cell viability of extract from roasted date seed showed high metabolic activity showed at 50, 500 and 1000 concentrations on day two.

Conclusion: The present study showed that roasted and non-roasted date seed extract were significantly reducing adipocyte differentiation compared to the control group. We observed that non-roasted date seed extract significantly reduces adipocyte with 100 µg/ml concentration and above.

Practical Implications: These results may have clinical implications for diabetic patients since date seed extract has anti-adipogenic, anti-oxidants, anti-bacterial and anti-viral effects. Further studies are needed to determine the correct dose of date seed extract.

Introduction

Pluripotent stem cell (PSCs) can proliferate and differentiate into different cells of the three germ layers. These two characteristics make PSCs an excellent source for generative medicine to treat various diseases and injuries. Adult Stem Cells (ASCs) are found in mature tissues such as bone marrow. Plasticity of ASCs can proliferate and generate lineages of different cells. That is why these cells are a good source for injuries repair and organ regeneration in various species [1]. Bone marrow Mesenchyme Stem Cells (MSCs) have been used in treating many diseases with varying degree of success including blood-related disorders, multiple sclerosis, immune deficiency disorders, cancer and many more [2]. Bone marrow MSCs are multipotent stem cells that have the ability to self-renew and differentiate into mesodermal cell lines under appropriate conditions into various cells different from the original cells. They can differentiate in vivo and in vitro into osteoblast, cardiomyoytes, adipocytes, myocytes, cardiomyocytes and more [3]. These cells play an important role in the maintenance of normal human body state. Differentiation of bone marrow MSCs should be balanced and regulated by a variety of hormones. Obesity is a metabolic disorder characterized by increase of adipocyte cells. Preventing obesity or decreasing weights intervention involve dietary restriction and increase physical activity. Therefore, understanding the differentiation of bone marrow MSCs into adipocytes in the presence of date seed extract will be investigated in this study as an alternative to inhibit adipogenesis [4]. Date seed extract have many priorities that can help in treating disorders effectively. One study has reported that date seed extract protects against hepatorenal toxicity. Another study showed that it also protects against focal cerebral ischemia [5]. Obesity is recognized as an epidemic and a concern to the global public health communities as it causes several chronic disorders associated with other comorbidities, which cause serious morbidity and mortality [6]. Recent studies shifted toward finding natural alternatives to reduce obesity rate. There is a body of literature defining the effects of natural remedies and dietary supplements to reduce blood glucose and thereby reducing fat [7]. A few studies executed by Prof. El Fouhil and his colleagues showed that date seed extraction has a positive effect on reducing blood sugar in rats with diabetes mellitus by reducing fat cells [8]. In this study, the effect of date seed extraction either roasted or non-roasted on inhibiting adipogenesis leading to reduce blood glucose. To achieve that, MSC"s were cultured and exposed to roasted and non-roasted date seed according to a specific protocol and conducted several tests to detect their viability and differentiation potential including Nile Red, Alamarblue, and Oil Red staining.

Objectives

The objectives of the present study are:

- To measure the effects of roasted and non-roasted date seed on adipogenesis.
- To determine what is the best way to cause adipogenesis that can significantly accelerate the proliferation rate of bone marrow MSCs in vivo.
- Detection of cell viability.
- Gene expression of key adipogenic genes including: AP2, AdipoQ, LPL, and PPARg.

Materials and Methods:

Cell culture

Bone marrow mesenchymal stem cell lines (human MSCS) is a gift from our collaborator from Denmark Professor Mousta Kassems lab in Odense. Cells were preserved in Dulbecco's Modified Eagle Medium (DMEM) that includes D-glucose 4500 mg/L, 110 mg/L sodium pyruvate, 4 mM L-glutamine, 10% fetal bovine serum (FBS), non-essential amino acids (Gibco-Invitrogen, USA), and 1x penicillin–streptomycin (Pen–strep). At 70-80% confluence, the media was changed to adipogenesis differentiation media containing; 115.65 ml DMEM (-), 15 ml FBS, 15 ml horse serum, 30 µL dexamethasone, 60 µL BRL, 45 µL insulin, 1500 µL IBMX, and 1500 µL Pen Strep. The media was changed every other day over 10 days. To elucidate, roasted date seed group had 3 plates of 48 well-plate and one plate of 24 well-plate; non-roasted had the same number of plates.

Each plate of the 48 well-plate was divided into seven groups as follows; CNT, 10, 50, 100, 500, 1000 and 5000 µg/ml of roasted or non-roasted date seed extract. Adipogenesis media was added on day zero with the date seed extract. For the first three days, one plate of the 48 well-plate from each group of the roasted and non-roasted date seed was used to measure cell viability through AB.

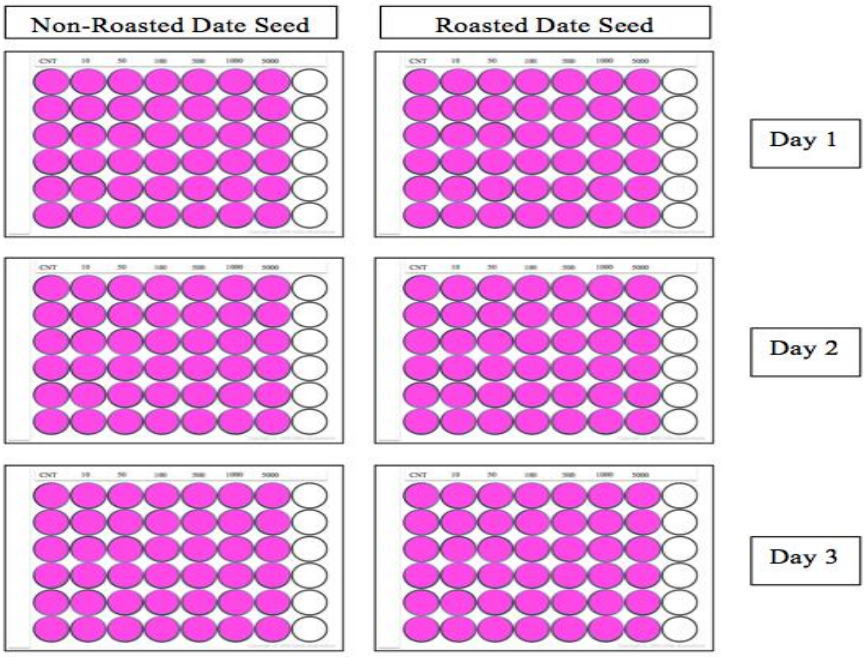

Figure 1 shows MSCs of six well-plates were divided between roasted and non-roasted date seed extracts used to measure AB on day 1, day 2 and day 3; (three each group with different concentrations including; CNT, 10, 50, 100, 500, 1000, and 5000). Adipocyte medium was added on day zero.

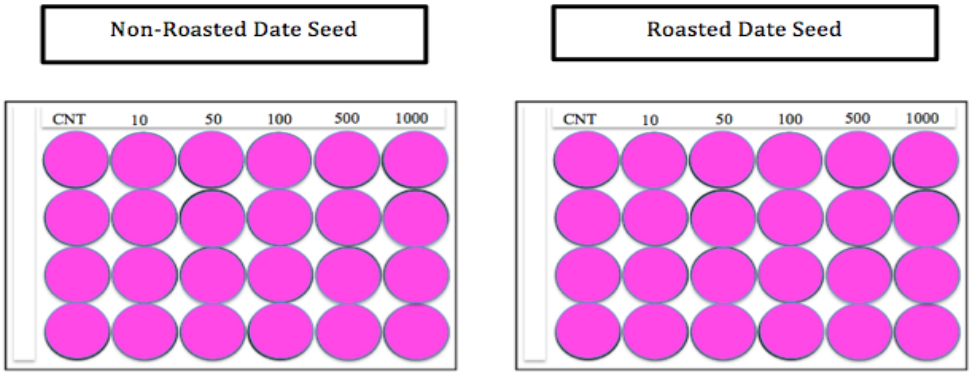

Figure 2 shows Two plates of MSCs were used to measure AlmarBlue and Nile Red. Adipogenesis medium with date seed extract (Roasted and Non-roasted) were added every other day; experiment terminated on day 9.

Date seed extraction

Seeds obtained from "Sukkary" dates; the first group of Non-roasted Date Seeds (NDS) were washed with water, dried and crushed in a mixer to become a powder. Then, it was mixed with water and put in a shaker with controlled temperature for 24 hours. A vacuum filtrations system with PES membrane of 0.22 µm was used to filter the solution from the big chunk of date seed. Then, a working concentration of NDS of 3.125 ml was diluted in 6.8 ml of water. The other group

of Roasted Date Seed (RDS) were washed, dried then roasted at 40℃ for about 20 minutes. Then, it was crushed in a mixer; it was also mixed with water and left in the shaker for 24 hours. It also was filtered using vacuum filtrations system with PES membrane of 0.22 μm. After that, a working concentration of 3.57 ml of RDS was diluted in 6.42 ml of water. In each group, we had a control group, and date seed extraction with a final concentration of 10 μL, 50 μL, 100 μL, 500 μL, 1000 μL and 5000 μL. A final concentration of NDS and RDS were mixed with adipogenesis solution as described above. The media was changed on day 2, 4 and 7.

Cell proliferation assay:

Cell growth was measured by the Nile Red, Alamar Blue, and Oil Red proliferation assay, which was performed on day 9 after induced differentiation. To make Nile Red staining, the sample were washed twice with PBS. A 75 μL of Nile Red was added in 15 ml of PBS. The plates were incubated for 10 minutes in the dark at room temperate. Then, MSCs were rinsed again with PBS. AlmarBlue and Oli Red were read using BioTek Synergy II microplate reader (BioTek Inc., Winooski, VT, USA) at Ex 485 nm/Em 572 nm. To make AlamrBlue, a 10% of the AlamarBlue® reagent will be added 90% of DEME (+) (for example, add 10 μL alamarBlue® reagent to 100 μL sample), followed by one hour incubation at 37ºC. The resulting fluorescence is read using BioTek Synergy II microplate reader (BioTek Inc., Winooski, VT, USA) at Ex 530 nm/Em 590 nm. To make Oil Red, it was diluted using deionized water with a proportion of 3:2 (i.e. add 12 ml Oil Red to 8 ml deionized water), before adding the solution, the plates were rinsed in PBS, then fixative was added and incubated at room temperature for 15 minutes. Finally, remove fixative and wash sample with deionized water three times.

Quantitative Real-time polymerase chain reaction (qRT-PCR):

The PureLink kit (Ambion by Life Technologies, USA, Cat No: 12183018A) was used for total RNA extraction as per the manufacturer protocol. Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific, USA) was used for total RNA was quantification. The High Capacity cDNA Reverse-Transcription kit (Applied Biosystem, USA) was used to generate complementary DNA (cDNA) from 1 μg of the RNA using the Labnet Multigene thermocycler. Relative expression levels of selected transcripts were measured using real-time PCR (Applied Biosystem-Real-Time PCR Detection System) and the Power SYBR Green PCR master mix (Applied Biosystem, UK) or with the TaqMan Universal Master Mix II, no UNG (Applied Biosystem, USA) according to the manufacturer's instructions. Differential expression was measured using a comparative Ct method and GAPDH as reference gene.

Statistical Analysis

All results are presented as the mean ± standard deviation (SD) from at least three independent experiments and P-values < 0.05 (using unpaired one-tailed t-test) were considered significant.

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Results

Cell Viability Assay of Roasted Date Seed

The viability of the cells grown has been evaluated using the AlmarBlue according to the standard protocol described. A total of six plates were divided between roasted and non-roasted date seed extracts. The cell viability of these plates was measured on day 1, day 2 and day 3 using AlmarBlue. Moreover, we measured the cell viability of the remaining two plates of the 24 well-plate on day 9 for cells incubated with different concentrations of roasted and non-roasted date seed extract with or

without adipocyte induction medium (Figure 6 in materials and method section). CNT. The intensity of fluorescence is proportional to the number of living cells in treated samples and CNT. AlmarBlue assay showed that roasted date extract manifested high cell viability on day 1 and day 2 compared to day 3 which showed a reduction in cell viability. The cell viability of extract from roasted date seed showed high metabolic activity showed at 50, 500 and 1000 concentrations on day two.

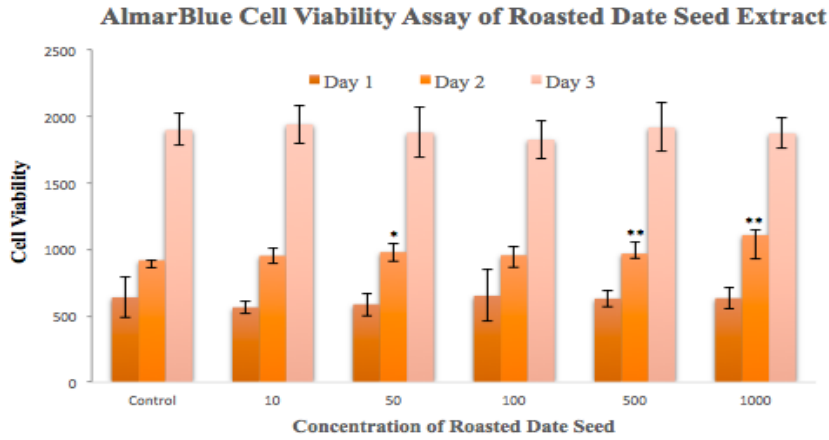

Figure 3 Cell viability and proliferation of MSCs treated with different concentrations of date seed extract. The first three days cells were incubated with different concentrations of roasted date seed extract.

Adipocyte Differentiation of extract from Roasted Date Seed

Roasted date seed extract resulted in significant increase of adipocyte differentiation at 1000 µg/ml concentration with a P.Value of (0.02) compared to the control group (P.Value = 0.13) and to the other concentrations (Table 1). The other concentrations of roasted date seed extract also exhibited a slight increase in the adipocyte differentiation, but it was not significant.

Table 1 Effects of roasted date seed extract on adipocyte differentiation

	Control	10	50	100	500	1000
AVG	100	95.32210919	95.048564359	103.4377701	98.75094808	111.0921539
STDV		4.447332328	9.165772152	3.327601184	3.113030009	3.42492188
P-value		0.137629352	0.26231589	0.140736894	0.313811324	0.022255415

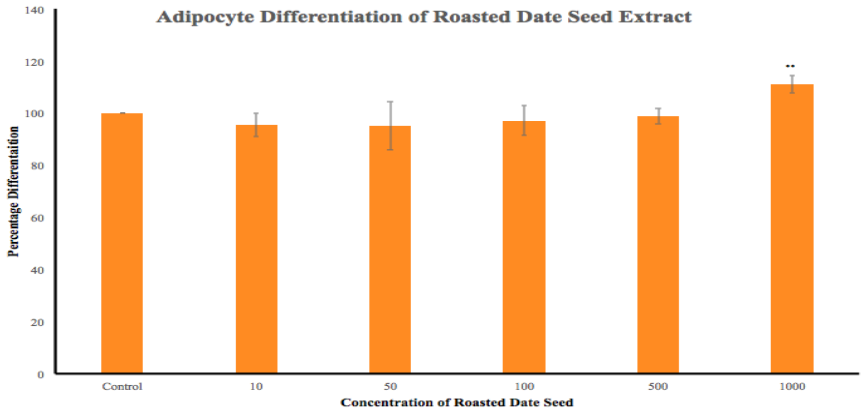

Figure 3 Adipocyte differentiation of roasted date seed extract among different concentrations on day 9 after using AlmarBlue (530 nm – 590 nm absorbance).

Non-Roasted Date Seed extract

Cell Viability Assay of Non-Roasted Date Seed extract

Human MSCs where cultured in non-roasted date seed extract and the viability showed significant increase at day two at concentration 10 and 50 µg/ml with P. value (0.0112 and 0.0117), respectively (Figure 9). At a concentration of 100, 500 and 1000 µg/ml concentration of non-roasted date seed extract, there was an increase in the cell viability with P. Value of (0.026, 0.04, and 0.027), respectively. AlmarBlue assay showed that non-roasted date extract manifested high cell viability on day one and day two compared to day three that showed reduced cell viability. Cell metabolic activity was highly significant at 10 and 50 concentrations on day 2.

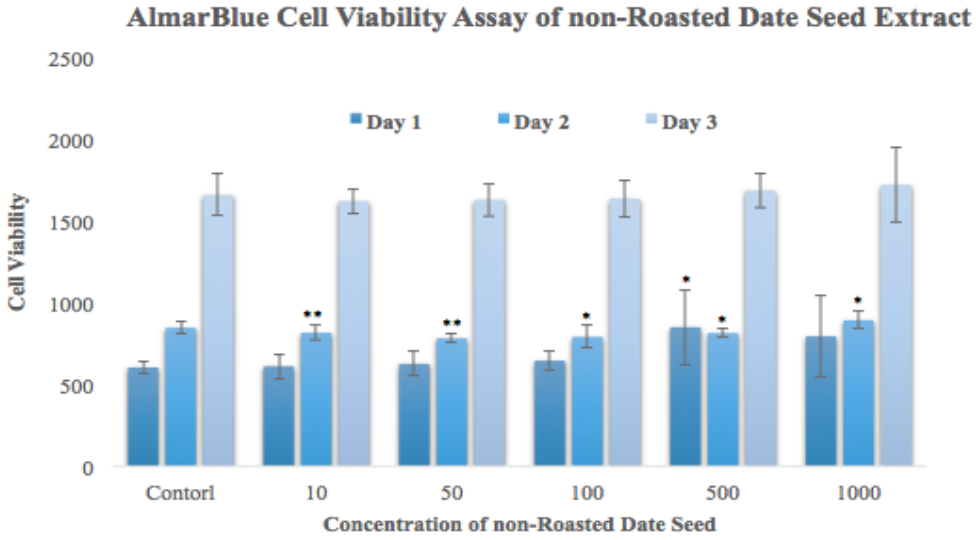

Figure 5 Cell viability among different concentrations on the first three days for cells incubated with different concentrations of non-roasted date seed extract with adipocyte induction medium and without CNT.

Adipocyte Differentiation of Non-Roasted Date Seed extract

Non-roasted date seed extract showed a reduction in the percentage of adipocyte differentiation at a higher concentration. At 500 µg/ml concentration of non-roasted date seed extract, there was a reduction of adipocyte differentiation with P-value (0.023). There was a significant reduction in the adipocyte differentiation at 1000 µg/ml concentration of non-roasted date seed. Other concentrations of non-roasted date seed showed increased in the percentage of adipocyte differentiation, but not significantly (Figure 10).

Table 2 shows effects of non-roasted date seed extract on adipocyte differentiation

	Control	10	50	100	500	1000
AVG	100	101.755835	101.8825733	92.15544792	78.7877932	77.59499617
STDV		0.489426661	4.892238432	3.950592542	8.298793903	1.030041493
P-value		0.012467306	0.286840984	0.03756835	0.023709976	0.000351892

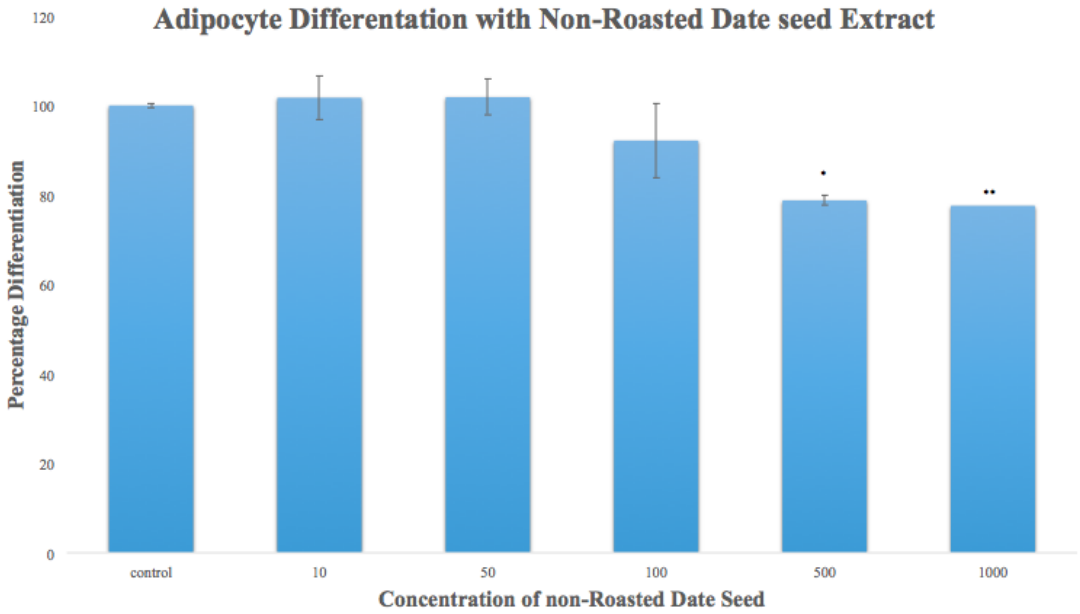

Figure 6 Shows adipocyte differentiation of non-roasted date seed extract among different concentrations on day 9 after using AlmarBlue assay (530 nm – 590 nm absorbance) and Nile Red assay (485 nm - 572 nm absorbance).

Gene expression of Cell Regulation Genes (AP2)

The gene expression of the cell regulation genes (AP2) in MSCs of roasted date seed showed downregulate effects concerning with the 10 and 50 µg/ml concentrations with a mean of (0.90 and 1.27) compared to 100, 500 and 1000 µg/ml concentrations with a mean of (1.91, 5.65, and 6.54) respectively. The foregoing indicates that low concentration of roasted date seed is better than the high concentration groups. Increase gene expression of AP2 leads to increase adipocyte; hence, increase the risk of obesity, T2DM and cardiovascular diseases.

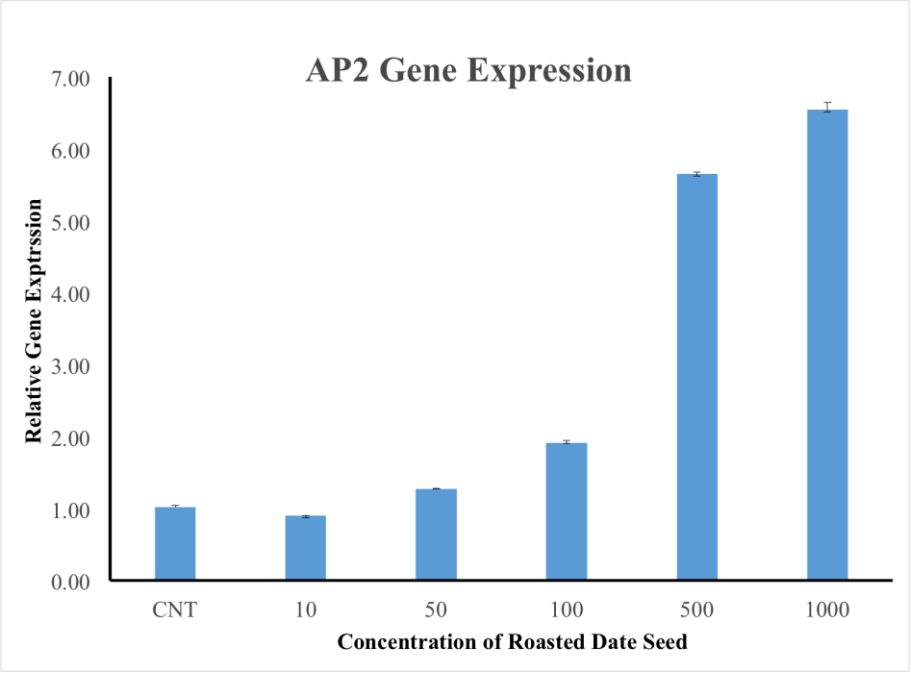

Figure 7 comparison of AP2 gene expression in MSCs in roasted date seed among different concentrations.

Gene expression of AdipoQ

Our statistical analysis of AdipoQ gene expression of roasted date seed showed in figure (12) the comparison between different concentrations of roasted date seed. Results showed decrease of gene expression mean in low concentration groups (10, and 50 µg/ml) with a mean of (0.79 and 1.00) compared to high concentrations group 100, 500, and 1000 µg/ml with a mean of (1.13, 2.83 and 3.82). A concentration of 50 µg/ml reduced adipocyte differentiation more than the control group (mean = 1.00), but not significantly. Therefore, we can say that the low concentration groups of roasted date seed is better than the high concentration groups in reducing fat tissue.

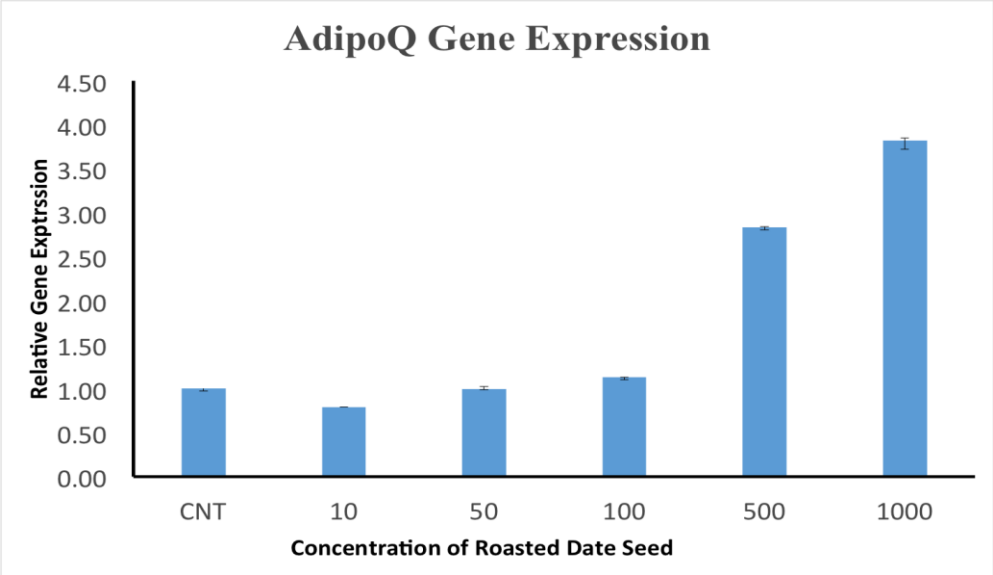

Figure 8 comparison of AP2 gene expression in MSCs in roasted date seed among different concentrations.

Gene Expression of non-Roasted Date Seed

Our statistical analysis of the gene expression (AP2) of non-roasted date seed showed downregulate effects concerning with 50 µg/ml concentrations with a mean of (0.58) compared to the other concentrations (Figure 15). The remaining concentrations showed high expression of the AP2gene. This indicates that low concentration of non-roasted date seed is better compared to the control group. Increase gene expression of AP2 leads to increase adipocyte; hence, increase the risk of obesity, T2DM and cardiovascular diseases.

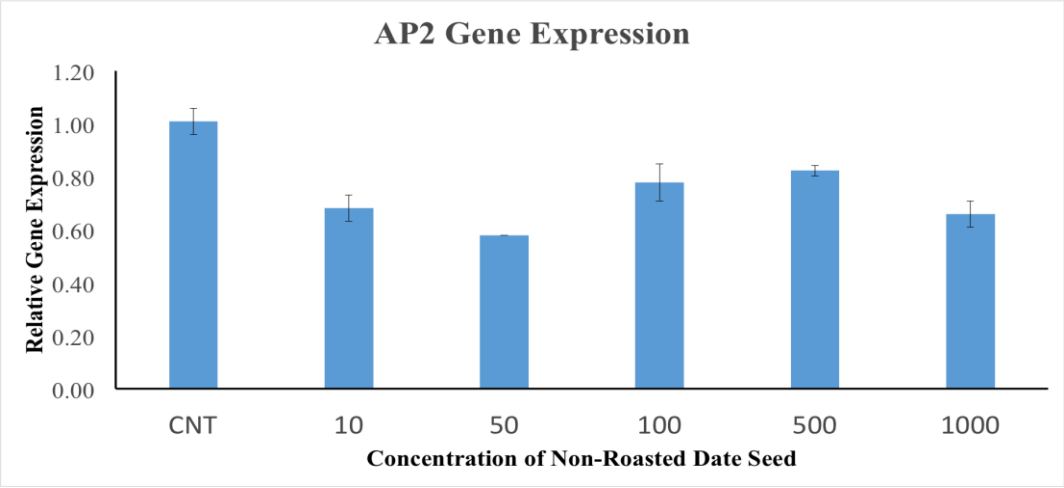

Figure 9 comparison of AP2 gene expression in MSCs in non-roasted date seed among different concentrations.

Gene expression of Cell Regulation Genes (AdipoQ)

AdipoQ gene expression of non-roasted date seed showed downregulate effects concerning all concentrations of non-roasted date seed compared to the control group. A concentration of 1000 µg/ml reduce adipocyte differentiation the most compared to the control group and other concentrations. It means that non-roasted date seed is better compared to the roasted date seed in reducing the fat tissue. Increase gene expression of AdipoQ leads to increase adipocyte; hence, increase the risk of obesity, T2DM and cardiovascular diseases.

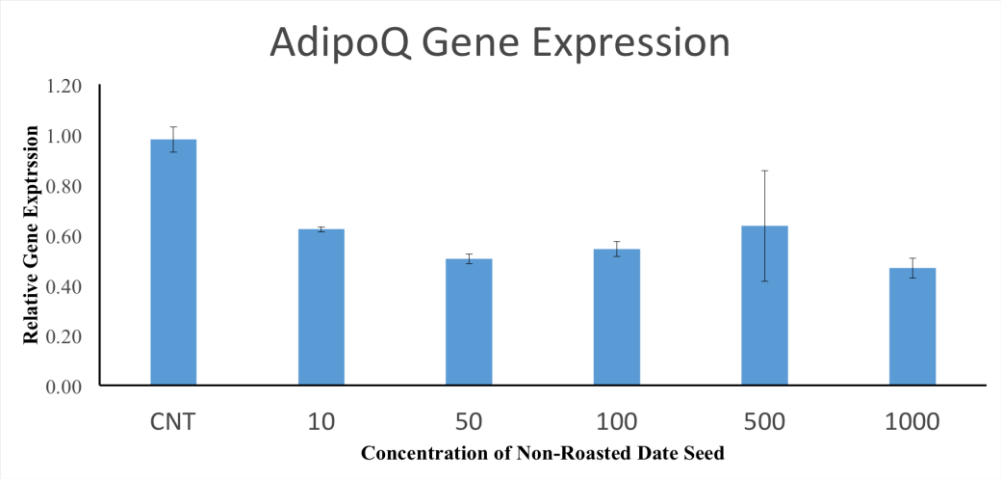

Figure 9 comparison of AdipoQ gene expression in MSCs in non-roasted date seed among different concentrations.

Discussion

Polyphenols is a natural element found in date seed and it has many properties that can combat chronic diseases such as diabetes, osteoporosis, cancer, neurodegenerative and cardiovascular diseases. It is believed that it inhibits adipogenesis and thereby reduces weight and metabolic disorders [10].Date seed is a rich source of polyphenol, which indicates a potential anti-adipogeneic and anti-hyperglycemic effects. Previous studies examined date seed extract effects and it showed that it has a reduction effect on the adipocyte differentiation and hence reducing blood glucose level [11]. PPARγ, the master regulator of adipogensis, has a role in inducing the adipogensis process with adiponectin and C/EBPα [12]. A variety of factors influence the adiopgensis process including the correct dose of date seed and the frequency of consuming date seed, which is required for the best therapeutic effects. The lowest effective dose of date seed extract used previously was 315 µg/ml [13].

In the current study, MSCs were treated with roasted and non-roasted date seeds extract, as the objective was to measure adipocyte differentiation among different concentrations [14]. Non-roasted date seed showed more significant results compared to roasted date seed extract. Non-roasted date seed extract reduced adipocyte differentiation among all different concentrations, especially at high concentration of 500 µg/ml and 1000 µg/ml. AlmarBlue cell viability assay of roasted date seed showed significant results with high concentrations only. These results indicated that date seed extract (roasted and non-roasted), with a significant result in the roasted date seed, induced adipocyte differentiation of MSCs compared to the control group. Our results demonstrated that non-roasted date seed extract was effective at a concentration of 1000 µg/ml [15].

Polyphenols inhibit adipogenes through inhibiting the signaling pathway of PPARγ, adiponectin and C/EBPα in the cell. PPARγ is a transcription factor that regulates adiponectin expression, which regulates metabolic activity through insulin sensitivity and fatty acid stimulation [16]. We observed there is an increase in gene expression of LPL and AdipoQ of non-roasted date seed indicating

dscrease of adipocyte differentiation [17-19]. Overall, gene expression of non-roasted date seed were low compared to roasted date seed. On the contrary, there is an increase in the gene expression of AP2 and AdipoQ of roasted date seed. Overall, gene expression of roasted date seed were high compared to roasted date seed [20-22].

Conclusion and Recommendations:

The present study showed that roasted and non-roasted date seed extract were significantly reducing adipocyte differentiation compared to the control group. We observed that non-roasted date seed extract significantly reduces adipocyte with 100 µg/ml concentration and above. These results may have clinical implications for diabetic patients since date seed extract has anti-adipogenic, antioxidants, anti-bacterial and anti-viral effects. Further studies are needed to determine the correct dose of date seed extract.

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