Anticancer activities of selected Emirati Date (*Phoenix dactylifera* L.) varieties pits in human triple negative breast cancer MDA-MB-231 cells

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**Abstract**

The date palm (*Phoenix dactylifera* L.) is an important fruit crop with significant pharmaceutical potential. Little data are available on comparative pharmaceutical importance of the date pits. We designed this study to assess the antitumorigenic effects of date palm pits extracts from different Emiratis varieties. We used MDA-MB-231 cells derived from triple negative breasts cancer tissues as a model. We found that out of the 17 date pits extracts from 6 Emiratis varieties, three (Khalas extract in water + acetone (1:1), Abu-Maan extract in MeOH + Chloroform (1:1) and Mabroom extract in water + acetone (1:1)) were found effectively cytotoxic and changed morphology of cells in dose and time dependent manner. We found the maximum effect at 2.5 mg/mL concentration at 72 h. We calculated IC50 values for these varieties at 24 h. IC50 values for Khalas, Abu-Maan and Mabroom were 0.982 mg/mL, 1.149 mg/mL and 2.213 mg/mL respectively. We treated the cells with IC50 values of extracts and observed changes in protein profile using human kinase array kit. After analyzing the results, we suggest that EGFR/ERK/FAK pathway, eNOS and src family proteins are targets of these extracts. We conclude that date pits extracts can be a possible therapeutic agent against cancer and we suggest further studies.

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1. Introduction

The date palm is among the top economic fruit tree grown in United Arab Emirates (UAE) and rest of the Arab Peninsula. The UAE is the fourth largest producer of dates in the world with 750,000 tons of the fruit produced annually, contributing to 14% of global output (Gul News, 2017). Though the date palm fruit serve as the low-cost food for millions of people which is rich in carbohydrates, proteins, amino acids, fibers, carotenoids, fatty acids, and a long range of minerals such as calcium, magnesium, potassium, iron, etc. (Al Farsi and Lee, 2008a,b; Biglari et al, 2009; Vayalil, 2012). The date pits also called date seeds or date stones contain different functional compounds like fibers, fat, proteins, moisture, vitamins and high phenolic compounds that make them an attractive target for different medical conditions (Al Farsi and Lee, 2008a,b). The high amount of dietary fibers in the pits suggest them to be effective in different medical conditions, like obesity, diabetes, hypertension, heart disease, intestinal disorders and in colorectal and prostate cancers (Kritchevsky, 1988; Johnson and Lee, 2008a,b). The date pits contain different functional compounds like fibers, fat, proteins, moisture, vitamins and high phenolic compounds that make them an attractive target for different medical conditions (Al Farsi and Lee, 2008a,b). The high amount of dietary fibers in the pits suggest them to be effective in different medical conditions, like obesity, diabetes, hypertension, heart disease, intestinal disorders and in colorectal and prostate cancers (Kritchevsky, 1988; Johnson and Southgate, 1994; Tariq et al., 2000; Varijazxhan et al., 2020). Phenolic compounds present in date pits especially phenolic acid and flavonoids are supposed to have antioxidant, anti-

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Peer review under responsibility of King Saud University.
tumorigenic and anti-mutagenic roles (Bailey and Williams, 1993; Liverio et al., 1994; Peterson and Dwyer, 1998).

Breast cancer is the most prevalent cancer among women with a wide range of etiological factors. Breast cancer is of four different types depending on the receptors including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2) and the type that lacks all three receptors is called triple negative breast cancer (TNBC) (Lehmann et al., 2011). MDA-MB-231 cells are used as model for TNBC cells that do not respond to hormone therapy, hence with limited treatment options. In breast cancer, GATA3, p53, Bax, p21, ELF5, CDKs genes involvement is reported to affect cell cycle, DNA damage repair or inducing apoptosis (Malumbres and Barbacid, 2009; Choi et al., 2009; Sun et al., 2016). Moreover, in vivo radioprotective role of date palm pits extract is also reported recently (Khezerloo et al., 2019). In this context, the current study is designed to screen 17 different extracts from 6 varieties of Emiratis date pits on MBA-MD-231 cells in order to know their potential to be used as a food supplement or treatment against the TNBC.

2. Materials and methods

2.1. Dates pits material

We purchased fruits of date palm varieties (Khalas, Abu Maan, Ajwa, Fard, Mabroom and Lulu) from Al FOAH Company, Al Saad, Al Ain, Abu Dhabi, United Arab Emirates (UAE) in good conditions free from any visible fungal infection or any other damage. The supplier's information about the different varieties are noted and used. The fruits samples were stored at 4 °C till further use.

2.2. Collection of pits

The pits were removed at Environmental Engineering laboratory, University of Sharjah. The pits were washed with deionized distilled water and dried at room temperature using cleaned filter papers. The pits were further crushed and ground into fine powder using a grinder (Kniftec 1095 laboratory mill) at 10,000 rpm.

2.3. Extracts preparation

We used different organic solvents, either alone or in combinations to obtain extracts from the stored date pits varieties powder (Table 1). Each date variety pits powder was soaked in selected organic solvent (20 g in 600 mL) and stirred gently at 25 °C for 24 h. The solvent extract was centrifuged, and the supernatant was evaporated to dryness in rotary-evaporators under vacuum at 50 °C. The dry remainder was collected and stored in air-sealed containers at 4 °C until further use. For preparation of stock solution, the extracts were first dissolved in 1% dimethyl sulfoxide (DMSO) and then distilled water was added to make the stock solution of 10 mg/mL. The working solutions for treatment of the cells were prepared for each variety pits extract by diluting it aseptically into the final concentration of 0.5 mg/mL, 1.0 mg/mL and 2.5 mg/mL.

2.4. Cell culture and experimental design

Human TNBC derived cell line MDA-MB-231 cells were cultured in 96 well plates at the density of 10,000 cells per well in Dulbecco's Modified eagle's Medium (DMEM) supplemented with 10%FBS and 1% Pen/Stept at 37 °C with 5% CO₂. The cells were incubated for 24 h to adhere and regain the original shape and then treated with three different concentrations (0.5, 1.0 and 2.5 mg/mL) of each variety of date pits extract in the following design as shown in flowchart in Fig. 1.

- Group 1: The cells were treated with 0.5 mg/mL extract for 24, 48 and 72 h respectively. The cells were photographed and MTT assay was performed at each time interval.
- Group 2: The cells were treated with 1.0 mg/mL extract for 24, 48 and 72 h respectively. The cells were photographed and MTT assay was performed at each time interval.
- Group 3: The cells were treated with 2.5 mg/mL extract for 24, 48 and 72 h respectively. The cells were photographed and MTT assay was performed at each time interval.
- Group 4: Separate untreated control group for each 24, 48 and 72 h.

2.5. MTT assay

The cells in the above-mentioned experimental design were put to MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay at 24, 48 and 72 h in order to know the time dependent cytotoxicity of the date pits extracts. After incubation for the respective period, the medium was removed, and the cells were washed with PBS and 20 μL of MTT solution was added to each well to achieve the final concentration of 0.5 mg/mL in 200 μL of medium. The cells were incubated for 4 h at 37 °C. After that, medium with MTT was removed and 200 μL of dimethyl sulfoxide (DMSO) was added to each well. The 96-well plate was shaken gently for 15 min in dark. The absorption was read at 570 nm wavelength using microplate reader (EPOCH Biotek). All experiments were repeated three times in triplicates and the results are shown as Mean ± SD. IC50 was calculated using online AAT Bioquest calculator for the extracts that followed time and dose dependent pattern.

2.6. Microscopic study

According to the mentioned experimental design, cells were photographed with 40x magnification using inverted phase contrast microscope (Olympus) attached with camera (OPTIKA Italy) and OpticalSview imaging software.

2.7. Phospho-protein array analysis

After analyzing results of MTT and morphological changes, three date pits extracts were selected for further investigation. The extracts i.e. Khalas (water + acetone 1:1), Abu Maan (methanol + chloroform 1:1) and Mabroom (water + acetone 1:1) were selected based on their cytotoxicity and morphological changes in MDA-MB-231 cells. The phospho-kinase array was per-
formed using the Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems). MDA-MB-231 cells were cultured at the concentration of $25 \times 10^5$ cells per well in 6 wells plate in triplicate for 24 h. After the cells were adhered and re-gained the original shape, these were treated with date pits extracts with IC50 concentrations i.e. 0.982 mg/mL, 1.149 mg/mL and 2.213 mg/mL for Khalas, Abu-Maan and Mabroom respectively for 24 h. After that medium was removed and cells lysates were prepared, and phosphorylated protein kinases were detected, according to the manufacturer’s protocol. Chemiluminescence was detected using ChemiDoc™ Gel Imaging System (Bio-Rad, USA). The intensity was measured using ImageJ software and converted into percent expression of each kinase.

3. Results

After assessing the cytotoxic activity of all the extracts, we chose three extracts based on their consistent time and dose dependent effect on MDA-MB-231 cells. The results for these are mentioned below. Rest of the extracts didn't show consistent, dose and time dependent effects and hence, data for these are provided in supplementary information S1.

3.1. Effects of the three datepalm varieties pits extracts on MDA-MB-231 cells viability

3.1.1. Khalas

The MDA-MB-231 cells were treated with three different concentrations of Kahals pits extracts (0.5, 1 and 2.5 mg/mL) for three different time points (24, 48 and 72 h). We found that Khalas pits extract in water and acetone (1:1) had no significant effect on the cell viability of MBA-MD-231 cells at 24 h. At 48 h, we found that at the highest dose i.e. 2.5 mg/mL the cell viability was significantly reduced (p < 0.05) to 30% compared not only to control but also to the rest of the lower doses. With the increase in time to 72 h the second highest concentration of 1 mg/mL also significantly reduced (p<0.05) the cells viability to 70% along with the highest concentration of 2.5 mg/mL upto 10%. This shows that Khalas variety pits affect the cells viability in higher doses with longer time points (Fig. 2A).

3.1.2. Abu-Maan

The MDA-MB-231 cells were treated with three different concentrations of Abu Maan pit extracts (0.5, 1 and 2.5 mg/mL) for three different time points (24, 48 and 72 h). We found that Abu-Maan pits extract in MeOH and Chloroform (1:1) reduced the MDA-MB-231 cells viability upto 40% at 24 h significantly (p < 0.05) at the highest concentration of 2.5 mg/mL compared to untreated control. At 48 h, we found that the two highest doses i.e. 1 mg/mL and 2.5 mg/mL significantly (p < 0.05) reduced the cell viability upto 50% and 15% respectively compared to untreated control. At 72 h, we found significant (p < 0.05) decrease in cell viability at 1 mg/mL and 2.5 mg/mL upto 40% and 10% respectively compared to the untreated control (Fig. 2B).

3.1.3. Mabroom

The MDA-MB-231 cells were treated with three different concentrations of Mabroom pits extracts (0.5, 1 and 2.5 mg/mL) for three different time points (24, 48 and 72 h). We found that Mabroom pits extract in water and acetone (1:1) had no significant effect on the cell viability of MBA-MD-231 cells at 24, 48 and 72 h at concentrations of 0.5 and 1 mg/mL, however significantly (p < 0.05) reduced the cells viability after treatment with 2.5 mg/mL at all time points. The viability of cells reduced upto 40% at 2.5 mg/mL at 24 h, 30% at 48 and 72 h compared to untreated control (Fig. 2C).

3.2. Effects of three datepalm varieties pits extracts on morphology of MDA-MB-231 cells

Phase contrast microscope was used to observe the morphological changes in the cells after treatment with the datepalm varieties pits extract. We observed morphological changes like cell shrinkage, reduced cell volume, detached and rounded morphology with increased number of floating cells (dead cells) of MDA-MB-231 after treatment with datepalm variety Khalas, Abu-Maan and Mabroom. The changes were more evident and clearer at the highest dose (2.5 mg/mL) at 72 h compared to lower doses and shorter period (24 and 48 h). The untreated control maintained the typical adherent cancer cell morphology (Fig. 3).

3.3. Phosphoproteomic pathway signature after treatment with selected extracts

To gain insight into the effects of pits extracts, we treated the MDA-MB-231 cells with IC50 values for the three extracts i.e. 0.982 mg/mL, 1.149 mg/mL and 2.213 mg/mL extracts of Khalas in water and acetone (1:1), Abu-Maan in methanol and chloroform (1:1) and Mabroom in water and acetone (1:1) varieties respectively for 24 h. Regulation of phosphoproteins and their associated signaling cascades mediated by these mentioned extracts in MDA-MB-231 cells were analyzed using the human phospho-kinase array. We found that the three mentioned extracts affected the expression of 17/43 kinases at different levels. These include EGFR, eNOS, ERK1/2, FAK, Fgr, Fyn, GSK3 alpha/beta, Lck, p53, p70S6, PRAS40 and YES (Fig. 4A–E).
Fig. 2. The figure represents the cytotoxic effects of the three date palm pits extracts on human triple negative breast cancer MDA-MB-231 cells. A) Date palm variety Khalas (water + acetone, 1:1) B) date palm variety Abu-Maan (MeOH + Chloroform, 1:1) C) date palm variety Mabroom (Water + Acetone, 1:1) pits extracts showed cytotoxic effects against the mentioned cells in dose and time dependent manner. The experiment was repeated three times in triplicate and the percent viability is shown in figure. Asterisks (*) shows statistical significance at p < 0.05 among the groups. a–d represent the four groups control, 0.5 mg/mL, 1.0 mg/mL and 2.5 mg/mL respectively. The respective alphabet shows statistical significance against the group.
4. Discussion

Date palms is a species with a wide range of varieties producing delicious fruits. Fruits pits of dates are considered less important and normally are thrown after eating the fruits. However, after knowing the chemical composition of the pits these are getting central position in the investigations. Dates pits are rich in nutritive substances like proteins, fats, oils, dietary fibers, minerals that make them not only important as a food product but also give them medicinal value in different ailments. The phenolic compounds and flavonoids make them more attractive with multiple effects on human health with their anti-oxidant, anti-inflammatory, anti-mutagenic, nephroprotective, hepatoprotective, anti-diabetic, anti-obesity and anti-cancer properties (Hossain et al., 2014; Masmoudi-Allouche et al., 2016; Khalid et al., 2017).

We found that extracts of three varieties (Khalas, Abu Maan, and Mabroom) of date palm have antiproliferative effects on MBA-MD-231 cells in dose and time dependent manner. In our screening, we studied 6 varieties of the date-palms which show that pits extract of all date varieties do not have same effects indicating difference in chemical composition of each variety. Earlier studies have also found antitumor activities of date (variety not mentioned) pits water extract in HCT-15 colon cancer cells (Sundar et al., 2017), and methanolic extracts of Arechti and Kobbi varieties of date palm in HepG2 and HeLa cells (Mansour et al., 2011; Thouri et al., 2019). Besides this, in vivo radioprotective and in vitro antiproliferative effects of date pits (variety not mentioned) n-hexane extract is reported in HepG2, A-549, and MCF-7 cells (Al-Sheddi, 2019; Khezerloo et al., 2019). However, the solvent of the extract and variety of dateplam used were different from our studies, which might have difference in severity as well as different effect on the type of cancer. Furthermore, oil derived from date palm variety Deglet-Nour also showed antiproliferative activity in HeLa cells. In the same study as antioxidant activity was reported for the same variety showing the possible reason for the anti-proliferative activity (Mansour et al., 2011). Antiproliferative effects of the three date palm varieties in the current study are supported by noticing the morphological changes (detached and round morphology, cell shrinkage, increased number of dead cells) in the cells after the series of treatments at three different time points. The figures clearly show large number of dead and detached cells indicating toxic effects of the date palm pits extracts on the MDA-MB-231 cells. It is supporting the idea that these mentioned extracts are affecting cell cycle, apoptosis and proliferation individually or all the three processes together. The use of crude extracts that are rich in phenolic and flavonoid contents (Hussain et al., 2019) in addition to dietary constituents like fats, proteins, carbohydrates and dietary fibers might be the possible reason for the multi-effects of the extracts. Abu-Maan and Khalas varieties are sharing antibacterial and antifungal activities as well as anti-proliferative activities, where Khodari and Ajwa showed good antimicrobial potential, however inconsistent anti-proliferative activity.

In order to have insight into the understanding of the date pits effects at molecular level, we used the human phosphor-kinase array kit for the three most potential varieties. We found a list of proteins (EGFR, ENOS, ERK1/2, FAK, FGR, FYN, GSK3β, LCK, P53, P70S6kinase, PLC-γ1, PRAS40, RSK1/2/3 and YES) which are down-regulated by Khalas, Abu-Maan and Mabroom varieties. EGFR induced activation of ERK and Akt is reported in MDA-MB-231 cells (Chun and Kim, 2013). In our results the date pits varieties suppressed the EGFR and ERK1/2, however Akt remained unaffected suggesting its affect through MAPK pathway and avoiding PI3/Akt pathway. Furthermore, FAK is a determinant in initiation, progression and metastasis of breast cancer (Luo and Guan, 2010) and is targeted by the mentioned extracts and works through ERK1/2 (Benzina et al., 2016). As eNOS expression is calcium dependent (Xu and Purtzki, 2010), therefore suppression of eNOS suggests that the pits extracts targeted the calcium pathway as well suggesting calcium dependent enhanced apoptosis. Fyn is an SRC family kinase involved in cell growth, death, morphogenic transformation and cellular motility of breast cancer cells (Elias and Ditzel, 2015). The members of the SRC family (Fyn, Fgr and...
oncogene, GSK-3-alpha–beta, and Lck) are reported to be upregulated in renal cell carcinomas (Qayyum et al., 2012) hence supporting our results in MDA-MB-231 cells. Interestingly MDA-MB-231 has high levels of a mutant p53 and phospholipase D (PLD) activity, which provides a survival signal in these cells when deprived of serum growth factors (Hui et al., 2006), however in our results, mutant p53 levels is suppressed by all these three extracts.

5. Conclusion

Three date varieties (Khalas, Abu-Maan and Mabroom) pits extracts have cytotoxic effects on MDA-MB-231 cells and change the morphology aggressively. The phosphokinase signature suggests that these extracts exhibit their effects through EGFR/ERK/FAK pathway, SRC family of kinases and calcium signaling. All these
needs further verification and confirmation individually using specific inhibitors and stimulators of the mentioned pathways.

Funding

A grant number (15020401002) from University of Sharjah, PI. Abdallah Shanableh, supports this study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2020.09.001.

References

Al-Farsi, M.A., Lee, C.Y., 2008. Nutritional and functional properties of dates: a review. Crit. Rev. Food Sci. Nutr. 48 (10), 877–887.
Al-Farsi, M.A., Lee, C.Y., 2008. Optimization of phenolics and dietary fibre extraction from date seeds. Food Chem. 108 (3), 977–985.
Al-Sheddi, E.S., 2019. Anticancer potential of seed extract and pure compound from Phoenix dactylifera on human cancer cell lines. Phcog. Mag. 15, 494–499.
Bailey, C.S., Williams, D.E., 1993. Potential Mechanisms for Food-related Carcinogens and Anticarcinogens. Food Technology, USA.
Benziina, S., Harquail, J., Guerrette, R., O'Brien, P., Jean, S., Crapoulet, N., Robichaud, G.A., 2019. Potential Mechanisms for Food-related Carcinogens and Anticarcinogens. Food Technology, USA.
Biglari, F., AlKarkhi, A.F., Easa, A.M., 2009. Cluster analysis of antioxidant compounds in dates (Phoenix dactylifera): Effect of long-term cold storage. Food Chem. 112 (4), 998–1001.
Chun, J., Kim, Y.S., 2013. Platycedrin D inhibits migration, invasion, and growth of MDA-MB-231 human breast cancer cells via suppression of EGR-mediated Akt and MAPK pathways. Chem. Biol. Interact. 205 (3), 212–221.
Elias, D., Ditzel, H.J., 2015. Fyn is an important molecule in cancer pathogenesis and regulating key biomarkers. OncoTargets Therapy 9, 21.
Hui, L., Zheng, Y., Yan, Y., Bargonetti, J., Foster, D.A., 2006. Mutant p53 in MDA-MB-231 breast cancer cells is stabilized by elevated phospholipase D activity and contributes to survival signals generated by phospholipase D. Oncogene 25 (55), 7305.
Hussain, M.I., Semreen, M.H., Shanableh, A., Khattak, M.N.K., Saadoun, I., Ahmady, I. M., Mousa, M., Darwish, N., Radeef, W., Soliman, S.S., 2019. Phenolic composition and antimicrobial activity of different emirati date (Phoenix dactylifera L.) pits: A comparative study. Plants 8 (11), 497.
Johnson, I.T., Southgate, D.A., 1994. Dietary Fibre and Related Substance. Chapman and Hall, London, UK.
Khalid, S., Khalid, N., Khan, R.S., Ahmed, H., Ahmed, A., 2017. A review on chemistry and pharmacology of Ajwa date fruit and pit. Trends Food Sci. Technol. 63, 60–69.
Khezerloo D, Mortezazadeh T, Farhood B, Sheikhzadeh P, Seyfizadeh N, Pezghman L. The effect of date palm seed extract as a new potential radioprotector in gamma-irradiated mice. J. Can Res Ther [serial online] 2019 [cited 2020 Sep 12];15:517–21. Available from: http://www.cancersciencejournal.net/text.asp?2019/15/3/517/231117.
Kritchekvsky, D., 1988. Dietary fiber. Annu. Rev. Nutr. 8, 301–328.
Pourzolati, P., Morazzoni, P., Bombardelli, E., 2019. Antimutagenic activity of procyanidins from Vitis vinifera. J. Study Med. Plants 65, 203–209.
Mansour, H.R., Hentati, B., 2016. Breast cancer malignant processes are regulated by Pax-5 through the disruption of FAK signaling pathways. J. Cancer 7 (14), 2035.
Sun, J., Guo, Y., Fu, X., Wang, Y., Liu, Y., Huo, B., Hu, X., 2016. Dendrobium candidum inhibits MCF-7 cells proliferation by inducing cell cycle arrest at G2/M phase and regulating key biomarkers. OncoTargets Therapy 9, 21.
Tariq, N., Jenkins, D.J., Vidgen, E., Fleschner, N., Kendall, C.W., Story, J.A., 2000. Effect of soluble and insoluble fiber diets on serum prostate specific antigen in men. J. Urol. 163, 114–118.
Vayali, P.K., 2012. Date fruits (Phoenix dactylifera Linn): an emerging medicinal food. Crit. Rev. Food Sci. Nutr. 52 (3), 249–271.
Xu, Z., Purtschi, M., 2010. Zinc depletion-induced apoptosis in human breast cancer MDA-MB-231 cells is calcium-dependent.