RESEARCH ARTICLE

Phytonutrient Effects of Date Pit Extract against Azoxymethane-Induced Oxidative Stress in the Rat Colon

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Abstract

Plants and their by-products offer a diverse mixture of chemical constituents like natural antioxidants. Date-pits are rich in phenolic compounds that have antioxidant potential. The main objective of this study was to investigate the protective effect of a date-pit extract (DPE) against AOM-induced colonic carcinogenicity and oxidative stress. Thirty-two weanling male Sprauge-Dawley rats were randomly divided into four groups (eight rats in each group). All rats were fed basic diet and water *ad libitum*, and randomly distributed per treatment groups as follows: negative controls injected with normal saline once a week for two weeks, a cancer group injected intra-peritoneally with azoxymethane (15mg/kg body weight) for two consecutive weeks, and DPE treated groups receiving the extract via the oral route (1.5ml/day) for the entire experiment in the presence or absence of AOM injection. Results showed that DPE contained phytonutrients that were capable of inhibiting chemically-induced oxidative stress in the rat colonic cells. In those animals that consumed DPE, a protective effect was observed against AOM-induced oxidative stress in rat colonic cells as evident by a significant decrease in MDA and oxidized DCF formation in AOM injected and DPE fed groups. It is concluded that DPE has potential antioxidant and anticarcinogenic properties.

Keywords: Date pit extract - oxidative stress - colon cancer - azoxymethane - total antioxidant capacity

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Introduction

Cancer is becoming the most important public health burden around the globe. The consumption of diet-containing carcinogens is associated with increasing risk of cancer in the colorectum, lung, prostate and breast, and it was documented that high consumption of plant-based foods might decrease the cancer risk (Abdull Razis and Noor, 2013). The protecting effects of plant-based foods and their byproducts have been attributed to their high amounts of phytonutrients that act as natural antioxidants against oxidative stress-mediated chronic disease, including cancer (Gurusak and DellaPenna, 1999).

Natural antioxidants are extensively studied for their capacity to protect organisms and cells from damage brought on by free radicals (Silva et al., 2007). As a matter of fact each cell is endowed by nature with adequate protective mechanisms against any harmful effects of free radicals, however, when it gets exposed to adverse physicochemical, environmental or pathological agents such as atmospheric pollutants, cigarette smoking, ultraviolet rays, radiation, toxic chemicals and over nutrients, this delicately maintained balance is shifted in favor of pro-oxidants resulting in oxidative stress (Devasagayam et al., 2004). Oxidative stress is considered a cause of numerous ageing and degenerative diseases (Cazzi et al., 1997) including colon cancer. Azoxymethane (AOM) acts as a colon cancer inducing agent in experimental rats and the mechanism is oxidative stress dependent (AnilKumar, 2010; Al-Numair et al., 2011; Waly et al., 2012).

Colon cancer is one of the most common malignancies in many regions of the world. The etiology of colon cancer is complicated and includes both genetic and environmental factors among the environmental factors; the dietary habits play a major role. Low intake of fibers, fruits and vegetables; and high intake of fat have been related with increased risk of colon cancer. It has been estimated that 30-40% of all tumors can be prevented with a correct lifestyle and diet, in particular colon cancer (Rajamanickam and Agarwal, 2008). Colon cancer is thought to arise from the accumulation of mutations in a single epithelial cell of the colon and rectum (Fearon and Vogelstein 1990), usually benign. However these changes, if remained untreated, some develop into cancer over time. It is recognized as the third most common cancer worldwide with high morbidity and mortality, and the fourth common cause of death (Sharma et al., 2014).

Fruits of the date palm are vital component of diet and commonly consumed in many parts of the world (Besbes

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et al., 2004). Date-pits contain 3.10-7.10% moisture, 2.30-6.40% protein, 5.00-13.20% fat, 0.90-1.80% ash, 22.50-80.20% dietary fiber, phenolics (3102-4430 mg gallic acid equivalents/100g) and antioxidants (580-929μm trolox equivalents/g) (Al-Farsi et al., 2007). Despite of that date pits are considered as waste and used mainly for animal feeds in the cattle, sheep, camel and poultry industries (Rahman et al., 2007). So it is pertinent to investigate the biochemical and phytochemical properties of date-pit in order to get best from the waste. Therefore, this study was planned to explore the antioxidants potential properties of date-pits and to assess the biological activity of this extract against Azoxymethane (AOM) induced-oxidative stress in rat colon.

Materials and Methods

Date-pits extract (DPE) preparation

Three kilograms of date-pits (Fardh variety from Al Batinah Region) were purchased from Al Shams Al Bahiya Factory, Barka, Oman, and were stored at room temperature (20-22ºC). The pits were washed and dried in air-drying oven (AFOS Mini Kiln, TecquipmentLimited, England) at 60ºC for 42h. The dried date-pits were ground in Foss Cemotec Grinder to form a powder and was stored in sealed containers at 22ºC. The dried powder was mixed with distilled water (10ml/g) and the mixture was stirred on a magnetic stirrer for three hours at room temperature (20-22ºC). The mixture was then filtered and the DPE obtained was stored at -40ºC until it was used for later experiments.

Experimental design and treatment protocol

Thirty-two weanling male Sprauge-Dawley rats at 8 weeks of age with an average body weight of 100±5g were used for this study. Rats were housed in galvanized iron cages in a temperature (23±1ºC) and humidity-controlled animal facility with a 12h light-dark cycle. They were randomly divided into 4 groups (8 rats per group) and were fed basic diet and water *ad libitum*. The control group did not receive any treatment (i.e., no azoxy methane (AOM-injection or saline). In the second group rats were given 2 intra-peritoneal injections of AOM (Sigma Chemical Co., St. Louis, MI) dissolved in physiological saline once a week (15 mg/kg body weight) for 2 weeks for a total of 30mg/kg body weight for each rat. The third group received oral date-pit extract (1.5ml/day) three times per week and the fourth group was AOM-injected group plus oral DEP administration. The extract dose was 1.05g fresh biomaterial per week, and DPE contained polyphenols 3.27±0.7mg gallic Acid/g dry date-pits. After 2 weeks from the last AOM injection, the animals were sacrificed by decapitation under diethyl ether anaesthesia after an overnight fast and the colon tissues were removed for subsequent analysis. The protocol used in this study was approved by Sultan Qaboos University (SQU) Animal Ethics Committee (SQU/AEC/2010-11/1).

Colon preparation and homogenization

The colons were carefully removed from rats, rinsed and approximately (~50mg) were immediately homogenized in 1ml of 100 mM potassium phosphate buffer (pH 7.2) by a glass-Teflon homogenizer and centrifuged at 6,000g at 4°C for 30min. The resulting supernatant was used for determination of protein, glutathione (GSH) and total antioxidant capacity (TAC), malondialdehyde (MDA) and dichlorofluoresceine fluorescence assay (DCF) measurements.

Analysis of protein content

Protein content of colon tissues was assayed by the method of Lowry et al. (1951), using bovine serum albumin as standard and protein content was expressed as mg/ml of sample.

Glutathione measurement

Aliquots of supernatant (100μl) were transferred to fresh Eppendorf tubes and 2 μL of monochlorobimane (25mmol/L) and 2μl of glutathione-S-transferase reagent were added, as provided by a commercial kit (Biovision, Mountain View, CA, USA, Catalog # K251). After 30min of incubation at 37ºC, the samples and standards were read in a fluorescence plate reader at 380/460nm. Glutathione (GSH) content was determined by comparison with values from a standard curve using freshly prepared GSH and normalized to the protein content of the assayed colon mucosal tissue homogenates.

Total antioxidant capacity measurement

A colorimetric method using Randox Assay Kit (Randox Laboratories Ltd, UK) was used to measure the Total antioxidant capacity (TAC). The assay is based on the incubation of samples with 2, 2'-azino-di-[3-ethylbenzthiazoline sulphonate (6)] diammonium salt (ABTS) with a peroxidase (methmyoglobin) and hydrogen peroxide to produce the radical cation ABTS+ which has a relatively stable blue-green color that is measured at 600nm. Antioxidants present in the assayed colonalmucossal tissue homogenate samples inhibit the oxidation of ABTS to ABTS+ (cause suppression of the colon production), and the conversion was proportional to their concentration. The capacity of the assayed samples antioxidants was compared with that of standard Trolox, a water soluble tocopherol analogue, which is widely used as a traditional standard for TAC measurement assays, and the assay results were normalized to the protein content of the assayed clonal tissue homogenates.

Evaluation of lipid peroxidation

Lipid peroxidation in the colon tissue homogenates was determined by measuring the production of malondialdehyde (MDA) using a commercial kit, (Thiobarbituric Acid Reactive Substances (TBARS) Assay Kit from Cayman Chemical Item Number 10009055). The TBARS Assay was conducted based on the manufacture instruction, and on brief MDA reacts with thiobarbituric acid (TBA) forming a coloured product measured at absorbance 532nm.

Dichlorofluorescein fluorescence assay

The dichlorofluorescein fluorescence (DCF) assay was used to measure cellular peroxide production and other

reactive species. Aliquots of colon samples were added to a medium containing Tris-HCl buffer (0.01 mM, pH 7.4) and dichlorofluoresceindiacetate (7µm). After the addition of dichlorofluoresceindiacetate, the medium was incubated in the dark for 1h until the fluorescence measurement (excitation at 488nm and emission at 525nm, with both slit widths at 1.5nm). Oxidized dichlorofluoresceine was determined using a standard curve of oxidized dichlorofluoresceine and results were expressed as µmol of oxidized DCF/mg protein.

Polyphenols analysis

Total phenolic compound was analysed using Folin-Ciocalteu method (Singleton and Rossi, 1965). In this method, 20µl of date-pits extract was placed into 10ml test tube and 250µl of Folin-Ciocalteu reagent and 750µl of 1.9 M sodium carbonate were added. The volume was made up to 5ml and test tube was placed on vertex equipment for one minute and then incubated for 2h in dark. The absorbance was then measured at 765nm using UV-visible Spectrophotometer. Gallic acid was used as a standard and a calibration curve was prepared using standard solution of Gallic acid. Results were expressed as Gallic acid equivalents (GAE) in mg/100g dry-extract. All experiments were done in triplicate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.03; GraphPad Software Inc. San Diego, CA). The results are expressed as means±standard deviation (SD) of 6 independent observations from each subgroup. The statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tukey's test and Student's unpaired t-test for means comparisons and a P-value of less than 0.05 was considered significant.

Results

Total phenols

Results of the water extract from date-pits showed that it contained polyphenol as 3.27±0.7mg GAE/g date-pits (41 mg/g dry-extract). Our results are in good agreement with (Al Farsi and Lee, 2008). They reported 25-55mg GAE/g phenols in dry-extract of date pits. In addition it was reported that the phenolic contents of date pits could be varied and reaching 21-62mg GAE/g date-pits based on the varieties and maturity stages (Awad et al., 2011; Benmeddour et al., 2013). In the case of date-pits, negligible data are available on the effect of varieties and maturity on the variations of phenolic contents.

Biochemical indices of colonic cells

Figure 1 represents the intracellular GSH measurements in colonic mucosal tissue homogenates in control, DPE, AOM and DPE plus AOM groups. AOM-injection caused GSH depletion (approximately 54%) as compared to control group, whereas DPE plus AOM group restored the depletion of GSH by 82%, though a non-significant difference ($p>0.05$) was observed between DPE supplement and control groups. GSH deficiency leads to oxidative stress, while elevated GSH levels increase antioxidant capacity and resistance to oxidative stress (Paul et al., 2011).

The total antioxidant capacity is presented in Figure 2. AOM caused a significant impairment in TAC activity as compared to the control group. DPE administration augmented TAC basal level and attenuated the AOM-induced TAC impairment as compared to AOM-injected groups. The observations are in line with the literature reports that documented the involvement of oxidative stress in the pathogenesis of colon cancer (Tsunadu et al., 2003, Waly et al., 2012) which correspondently reduced aberrant crypt foci (ACF) number.

The antioxidant properties of DPE are selectively manifested in the colonic cells of the AOM-treated rats as evident by the statistically significant ($p<0.05$) reduction in lipid peroxidation (MDA level) and by the observed trend for reduced protein oxidized products (oxidized DCF).

Figure 3 shows the peroxidation of lipid as determined

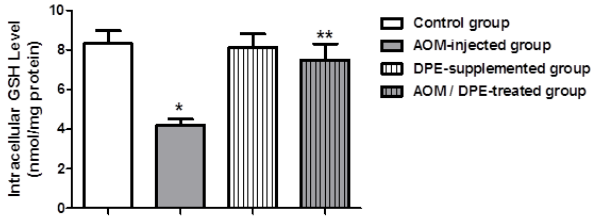

Figure 1. Date Pit Extract (DPE) Suppressed the AOM-Induced GSH Depletion in Rat Colonic Cells. *significantly lower as compared to control group, **significantly higher as compared to AOM-injected group, *p<0.05, **p<0.01. based on one way ANOVA analysis followed by Tukey's test

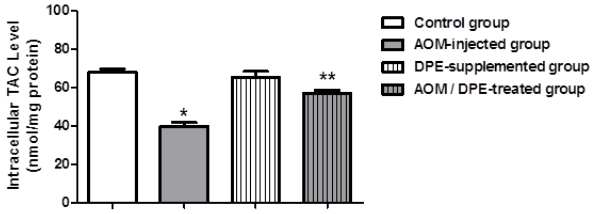

Figure 2. DPE Attenuated the AOM-induced TAC Suppression in Rat Colonic Cells. *significantly lower as compared to control group, **significantly higher as compared to AOM-injected group, *p<0.05, **p<0.01. based on one way ANOVA analysis followed by Tukey's test

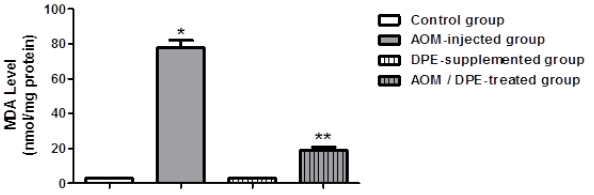

Figure 3. Lipid Peroxidation as Determined by MDA Level in Rat Colonic Cells. *significantly higher as compared to control group, **significantly lower as compared to AOM-injected group, *P<0.05, **P<0.01.based on one way ANOVA analysis followed by Tukey's test

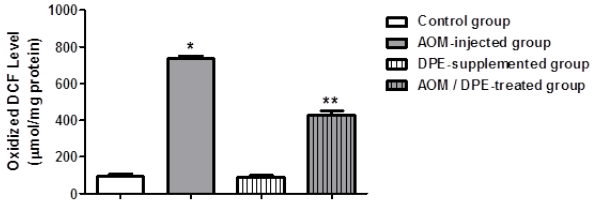

Figure 4. Peroxide Protein Formation as Determined by Oxidized DCF Level in Rat Colonic Cells. *significantly higher as compared to control group, **significantly lower as compared to AOM-injected group, *p<0.05, **p<0.01. based on one way ANOVA analysis followed by Tukey's test

by MDA level in colonic cells. Lipid peroxidation in DPE-supplemented group was significantly lower as compared to AOM-injected group. Peroxide protein formation was determined by oxidized DCF level in rat colonic cells, as shown in Figure 4. It was also significantly lower ($p<0.05$) in DPE-supplemented group as compared to AOM-injected group.

Discussion

Several *in vitro*, and *in vivo* studies supported that plants and their byproducts have strong chemo preventive ability against several neoplasm including colorectal cancer. The results of this study indicated that the AOM-injected rats showed significantly lower GSH, TAC and higher lipid peroxidation and peroxide protein formation as compared to control group. These findings suggest that adopting a specific DPE dietary regimen might reduce the AOM-induced effects on GSH and TAC levels in colonic mucosal tissue homogenates. The phenolic composition and antioxidant capacities of date palm fruit are affected by varieties and maturity stages (Awad et al., 2011; Benmeddour et al., 2013), while no data is available about date-pits. Azoxymethane (AOM) specifically induced oxidative stress and carcinogenesis on colonic cells, and specific dietary bioactive agents protected against AOM-induced oxidative stress and colon carcinogenesis in animal models (Waly et al., 2012). In consistent with this line of research, this study highlighted a specific protective role for DPE intake by abrogating the AOM observed suppression effects on antioxidant markers.

It has been hypothesized that oxidative stress induces the formation of lipid peroxides and other reactive oxygen species that play an important role in cancer pathogenesis (Baskar et al., 2012; Hamed et al., 2012) Lipid peroxidation is a free radical mediated process. Initiation of a peroxidative sequence is due to the attack by any species, which can abstract a hydrogen atom from a methylene group (CH2), leaving behind an unpaired electron on the carbon atom (•CH). The resultant carbon radical is stabilized by molecular rearrangement to produce a conjugated diene, which then can react with an oxygen molecule to give a lipid peroxyl radical (LOO•). These radicals can further abstract hydrogen atoms from other lipid molecules to form lipid hydroperoxides (LOOH) and at the same time propagate LP further.

Products of lipid peroxides result in the formation of highly reactive products such as malondialdehyde, which can bind to cellular proteins leading to pleiotropic effects and mutagenicity (Eoff et al., 2009). Malondialdehyde (MDA) formation is indicative of cellular lipids peroxidation and acts as a mutagen and carcinogen, MDA is highly reactive and capable of reacting with nucleic acids, proteins and phospholipids. Putative MDA adducts were found to accumulate in various cancer cell lines and enhances the production of skin tumors in mice model (Pizzimenti et al., 2013; Zhang et al., 2013).

Oxidative damage to cellular proteins causes amino acids modification and peptides fragmentation (Bozaykut and Sozen, 2013). Oxidative stress insults increased oxidation of thiol groups of different proteins, and subsequent proteins modification such as enzymes inactivation and genes silencing (Diers et al., 2013). The peroxidation reaction can be terminated by a number of reactions. The major one involves the reaction of LOO• or lipid radical (L•) with a molecule of antioxidant such as vitamin E or α-tocopherol (α-TOH) forming more stable tocopherol phenoxyl radical that is not involved in further chain reactions. This can be recycled by other cellular antioxidants such as vitamin C or GSH.

The present study found that DPE function as an antioxidant as well as displaying some anticarcinogenic potential. It is possible that DPE could be functioning by suppressing AOM by being present in the target tissue, leading to a reduction in colon cancer risk. Colon cancer is a highly prevalent cancer and treatment is costly, therefore prevention strategies are crucial for primary prevention of this chronic disease. Observations from this study indicate that DPE intake might provide protection against colon tumoriogenesis.

In conclusion, evidence from this study show that DPE is bioavailable and gets distributed to colonic cells in rats and acts as an in-vivo antioxidant as seen by the reduction in lipid peroxidation and protein oxidation. Although the mechanism of action for DPE is not fully understood, but DPE was found to contain phytonutrients compounds capable of inhibiting chemically-induced oxidative stress in the rat colonic cells.

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References

Abdull Razis AF, Noor NM (2013). Cruciferous vegetables: dietary phytochemicals for cancer prevention. *Asian Pac J Cancer Prev*, **14**, 1565-70.

Al-Farsi MA, Lee CY (2008). Optimization of phenolics and dietary fibre extraction from date seeds. *Food Chemistry*, **108**, 977-85.

Al-Farsi M, Alasalvar C, Al-Abid M, et al (2007). Compositional and functional characteristics of dates, syrups, and their by-products. *Food Chemistry*, **104**, 943-47.

Al-Numair KS, Waly, MI, Ali A, et al (2011). Dietary folate

protects against azoxymethane induced aberrant crypt foci development and oxidative stress in rats. *Exp Bio Md*, **236**, 1005-11.

Anilkumar KR, Sudarshankrishnan KP, Chandramohan G, et al (2010). Effect of aloe vera gel extract on antioxidant enzymes and azoxymethane induced oxidative stress in rats. *Ind J Exper Bio*, **48**, 837-42.

Awad MA, Al-Qurashi AD, Mohamed, SA (2011). Antioxidant capacity, antioxidant compounds and antioxidant enzyme activities in five date cultivars during development and ripening. *Scientia Horticulturae*, **129**, 688-93.

Baskar R, Lee KA, Yeo R, et al (2012). Cancer and radiation therapy: current advances and future directions. *Int J Med Sci*, **9**, 193-9.

Benmeddour Z, Mehinagic E, Le Meurlay D, et al (2013). Phenolic composition and antioxidant capacities of ten algerian date (phoenix dactylifera L.) cultivars: A comparative study. *J Funct Foods*, **5**, 346-54.

Besbes S, Blecker C, Deroanne C, et al (2004). Date seeds: Chemical composition and characteristic profiles of the lipid fraction. *Food Chemistry*, **84**, 577-84.

Bozaykut P, Sozen E (2013). The role of heat stress on the age related protein carbonylation. J Proteomics, *J Proteomics*, **89**, 238-54.

Cazzi R, Ricardy R, Aglitti T, et al, (1997). Ascorbic acid and b-carotene as modulators of oxidative damage. *Carcinogenesis*, **18**, 223-28.

Devasagayam TPA, Tilak JC, Boloor KK, et al (2004). Free radicals and antioxidants in human health:current status and future prospects. *JAPI*, **52**, 794-803.

Diers AR, Broniowska KA, Hogg N, et al (2013). Nitrosative stress and redox-cycling agents synergize to cause mitochondrial dysfunction and cell death in endothelial cells. *Redox Biology*, **1**, 1-7.

Eoff RL, Stafford JB, Szekely J, et al (2009). Structural and functional analysis of Sulfolobussolfataricus Y-family DNA polymerase Dpo4-catalyzed bypass of the malondialdehyde-deoxyguanosine adduct. *Biochemistry*, **48**, 7079-88.

Grusak MA, DellaPenna D (1999). Improving the nutrient composition of plants to enhance human nutrition and health. *Annu Rev Plant Physiol Plant Mol Biol*, **50**, 133-61.

Hamed EA, Zakhary MM, Maximous DW (2012). Apoptosis, angiogenesis, inflammation, and oxidative stress: basic interactions in patients with early and metastatic breast cancer. *J Cancer Res Clin Oncology*, **138**, 999-1009.

Lowry OH, Rosebrough NJ, Fait AL, et al (1951). Protein measurement with folin -phenol reagent. *J Bio Che*, **193**, 265-75.

Paul R, Prasad M, Sah N (2011). Anticancer biology of azadirachtaindica L (neem) A mini review. *Cancer Bio Therapy*, **12**, 467-76.

Pizzimenti S, Ciamporcero E, Daga M, et al (2013). Interaction of aldehydes derived from lipid peroxidation and membrane proteins. *Frontiers Physiology*, 4, 242.

Rahman MS, Kasapis S, Al-Kharusi NSZ, et al (2007). Composition characterisation and thermal transition of date pits powders. *J Food Engineering*, **80**, 1-10.

Rajamanickam S, Agarwal R (2008). Natural products and colon cancer: current status and future prospects. *Drug Devel Res*, **69**, 460-71.

Rodriguez-Ramiro I, Ramos S, Lopez-Oliva E, et al (2011). Cocoa rich diet prevent azoxymethane induced colonic preneoplastic lesions in rats by restraining oxidative stress and cell proliferation and inducing apoptosis. *Mol Nutr Food Res*, **55**, 1895-9.

Saafi EB, El Arem A, Issaoui M, et al (2011). Phenolic content and antioxidant activity of four date palm(Phoenix dactylifera L.) fruit varieties grown inTunisia. International *J Food Sci Technol*, **44**, 2314-9.

Silva E, Souza J, Rogez H, et al (2007). Antioxidant activities and polyphenol content of fifteen selected plant species from the Amazonian region. *Food Chem*, **101**, 1012-18.

Waly MI, Ali A, Guizani N, et al (2012). Pomegranate (*Punica granatum*) peel extract efficacy as a dietary antioxidant against azoxymethane-induced colon cancer in rat. *Asian Pac J Cancer Prev*, **13**, 4051-55.

Zhang X, Feng M, Liu F, et al (2013). Subacute oral toxicity of BDE-15, CDE-15, and HODE-15 in ICR male mice: assessing effects on hepatic oxidative stress and metals status and ascertaining the protective role of vitamin E. *Envi SCI Pollu Res Int*, 1-12.