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The Effect of Antioxidants in Medjool Variety of Date Fruits (*Phoenix dactylifera* L.) At the Tamr Stage on Oxidative Stress in Sprague-Dawley Rats Fed a High Fat Diet

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Abstract: The objective of this study was to investigate the effect of antioxidants in "Medjool" variety of Date Fruits (DF) (*Phoenix dactylifera* L.) at the tamr stage on oxidative stress in Sprague-Dawley rats fed a High Fat Diet (HFD). Proximate nutrient composition of the "Medjool" DF was determined as well as antioxidant capacity, total phenolic content and total flavonoid content and individual phenolic compounds gallic acid, p-hyroxybenzoic acid, vanillic acid and caffeic acid. The Sprague-Dawley rats were fed either control or HFD based on the AIN-93 diet for six weeks. They were then grouped as control, control+10% DF, HFD (45% kilocalories) and HFD+10% DF with 10 rats per group. The antioxidant capacity of DF was 1.55±0.058 mmole/100 g FW, the total phenolic content was 104.3±3.46 mg GAE/100 g FW and the total flavonoid content was 8.10±0.19 mg CEQ/100 g FW. Findings also showed that hepatic TBARS levels were significantly the highest in the HFD group (21.83±1.9 nmol mL^{-1} MDA equivalents) ($p<0.01$). The HFD caused a percentage increase of TBARS by 59.4% from the control group. The control+DF group had the highest level of serum total antioxidant capacity (0.164±0.017 mM Trolox equivalents) than the rest of the groups but the p-value only indicated a trend toward significance ($p = 0.082$). Dietary intake of "Medjool" variety of DF could have a potential role in ameliorating oxidative damage during obesity to possibly reduce the risk of obesity co-morbidities.

INTRODUCTION

The prevalence of obesity and its associated cardiometabolic disorders is rapidly increasing worldwide. This chronic, multifactorial condition is the sixth primary risk factor for the predisposition and the pathogenesis of several diseases including type 2 diabetes mellitus, atherosclerosis and certain types of cancer[1]. According to the WHO[2], at least 2.8 million people die each year for being overweight or obese.

Obesity is a subclinical inflammatory state which induces the progression of adipose tissue proinflammatory cytokines and adipokines[3], leading to insulin resistance and further oxidative damage[4]. The antioxidant system in

the body becomes overridden in this situation[5] which portrays the need for antioxidants from external sources.

Oxidative stress in obesity is derived from many possible mechanisms including chronic inflammation, hyperglycemia, increased tissue lipid levels, decreased antioxidant defenses, endothelial ROS production, hyperleptinemia and elevated muscular respiration rate[6]. Fruits and vegetables are a rich source of dietary antioxidants such as flavonoids, carotenoids and polyphenols which are considered for their antioxidant, antimutagenic and anti-inflammatory effects[7]. The bioactive compounds and micronutrients in fruits and vegetables contribute to an enhanced antioxidant defense system, attenuated oxidative stress and inflammatory markers and are therefore, highly recommended for consumption[8].

The antioxidants in DF is contributed to its content of phytochemicals such as carotenoids, polyphenols and flavonoids as well as the presence of vitamins and minerals that act as antioxidants like vitamin E and selenium[7, 9]. These antioxidants play a role in reducing oxidative stress by decomposing peroxides, preventing chain initiation and enhancing reductive capacity and radical scavenging[10]. This inhibits the oxidation of LDL and VLDL[11], DNA oxidation, platelet aggregation and improves low-grade inflammation[12] which decreases the risk of morbidity and mortality from coronary heart disease, stroke, certain types of cancer[13] and neurodegenerative diseases[12].

Several studies examined the antioxidant power of various fruits[14-16], however, to the best of our knowledge, no studies are available to determine the effect of DF, incorporated as part of the diet, on oxidative stress in humans and animals fed a High Fat Diet (HFD). Thus, the objective of this study was to identify the effect of the antioxidants in "Medjool" variety of DF on the levels of oxidative stress and antioxidant capacity in Sprague-Dawley rats fed a HFD compared to the control.

MATERIALS AND METHODS

Plant material preparation: A quantity of "Medjool" DF from the tamr stage of maturity was obtained from the local market in Jerusalem, Palestine.

Proximate analysis and antioxidant analysis of Date fruit: Proximate nutrient composition including moisture, crude ash, crude protein, crude fat and crude fiber was determined according to the Association of Official Analytical Chemists methods. For each extraction, duplicate samples were carried out. Nitrogen-free extract content which represents the soluble carbohydrates was calculated by difference.

Extraction of phenolic and flavonoid compounds from DF samples was undertaken as described by Odeh *et al*.[17]. Antioxidant capacity, total phenolic content and total flavonoid content were measured using Ferric Reducing Antioxidant Power (FRAP) assay as described by Benzie and Strain[18], Folin-Ciocalteu assay as described by Singleton and Rossi[19] and aluminum chloride colorimetric assay as described by Kim *et al*.[20], respectively. Individual phenolic compounds including gallic acid, p-hyroxybenzoic acid, vanillic acid and caffeic acid were measured using HPLC[17]. Assays were performed in triplicate on three DF extracts of methanol.

Animals: The 47 male, adult Sprague-Dawley rats weighing between 200-300 g were obtained from the Jordan University of Science and Technology Animal Unit, Irbid, Jordan. Animals were housed individually in plastic cages with stainless steel wire-mesh bottoms (North Kent Plastic Cages Ltd., England) under controlled temperature (24±2°C) and controlled light conditions (12 h light/dark cycle). Rats were acclimatized for one week before the beginning of the experiment by being fed stock diet and tap water *ad libitum*. The rats were randomly divided according to their weights into 5 groups. Group 1 was sacrificed as baseline. Serum and liver homogenates were collected and stored in the freezer for later analysis. The experimental groups were fed the prepared diet for six weeks *ad libitum*. Total food intake and body weights were measured once a week.

Experimental diet mixtures: Four types of experimental diet mixtures were adapted from the Guidelines of the American Institute of Nutrition 1993 for adult animals (AIN-93M) recommended by Reeves[21]. The experimental diets were: control diet (AIN-93M), control+DF diet (10% of diet from DF), HFD (45% kilocalorie (kcal) from fat) and HFD+DF (45% kcal from fat and 10% DF). Table 1 illustrates the formulation of the diet mixtures used in the experiment. Approximately 45% of total calories came from fat in the HFD[22]. Fresh DF preparation was incorporated into the diet mixture as 10 g/100 g diet[14]. Diet mixtures were isocaloric[23].

Date fruit preparation for experimental df diet mixtures: Fresh "Medjool" DF were pitted and ground (Matsushita elec. iND. Co. LTD, Japan) prior to diet preparation. DF and cornstarch were mixed using hands and allowed to pass through a sieve of 1 mm mesh to achieve homogenous mixtures[16]. Specific amounts of these mixes were used in the diet mixtures accordingly.

Blood samples collection: After 6 weeks, the animals were fasted overnight for 14 h, anesthetized by chloroform and sacrificed. Blood samples were collected from the right ventricle of the heart and serum samples were obtained by centrifugation at 3000 rpm for 20 min (HERMLE Z200A, Labortechnik, Wehingen,

Table 1: Formulation of the diet mixtures used in the experiment

Diet/	Control		Control+DF		HFD		HFD + DF	
Ingredients	g kg^{-1}/dt	Kcal	g kg^{-1}/dt	Kcal	g kg^{-1}/dt	Kcal	g kg^{-1}/dt	Kcal
Cornstarch	620.69	2482.768	620.69	2482.768	275.19	1100.76	275.19	1100.76
Casein[1]	140.	560	137.41	549.64	140	560	137.41	549.64
Sucrose	100	400	32.18	128.72	100	400	32.18	128.72
Soybean Oil	40	360	39.72	357.48	40	360	39.72	357.48
Fiber2	50	0	43.69	0	50	0	43.69	0
Mineral Mix[3]	35	29.37	35	29.37	35	29.37	35	29.37
Vitamin Mix[3]	10	38.98	10	38.98	10	38.98	10	38.98
L-cystine	1.8	0	1.8	0	1.8	0	1.8	0
Choline bitartrate[4]	2.5	0	2.5	0	2.5	0	2.5	0
TBHQ (mg)	8.0	0	8.0	0	38.71	0	38.71	0
Sheep Tallow	0	0	0	0	153.56	1382	153.56	1382
DF	0	0	100	284.2	0	0	100	284.2
Total	1000	3871	1000	3871	1000	3871	1000	3871
Kcal g^{-1} diet	3.871	3.871	3.871	3.871				

[1]Casein (= 85% protein); [2]Fiber (a -cellulose); [3]Mineral and vitamin mixes were prepared according to Reeves (1997); [4]Choline bitartrate (41.1% choline); dt = diet; DF = Date Fruit; HFD = High Fat Diet; TBHQ = Tert-Butylhydroquinone; Kcal = Kilocalorie

Germany)[16]. Afterwards, serum samples were stored in aliquots at -80°C until analyzed. Livers were removed and immediately washed with saline solution (0.9% NaCl), weighed and frozen at -80°C for later analysis[16].

Biochemical analysis of oxidative stress and antioxidant capacity: Biochemical analyses of oxidative stress and antioxidant capacity were conducted at Hamdi Mango Research Center, the University of Jordan, Amman, Jordan. All samples were tested in duplicate. Liver tissue TBARS of rats was determined using a commercial TBARS assay kit (Lot No. 313034; Zeptometrix Corporation, USA). MDA, a byproduct of lipid peroxidation[24], forms a 1:2 red adduct with Thiobarbituric Acid (TBA) was measured by spectrophotometry (Perkin Elmer Lambda 3, Canada) at 532 nm[25]. Serum total antioxidant capacity of rats was measured using antioxidant assay kit (Lot No. 0465494, Cayman Chemical, USA) as described by Miller *et al.*[26]. The inhibition of the oxidation of ABTS 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to $ABTS^{\cdot+}$ was measured spectrophotometrically at 405 nm (ELx 808™ Absorbance Microplate Reader, BioTek Instruments Inc., USA).

Statistical analysis: Statistical analysis of the data was performed using Statistical Package for the Social Sciences (SPSS) for windows (IBM®, SPSS for windows Version (20) 2010, Chicago). Data were expressed as mean±SEM. Statistical significance for differences in variable means between study groups were assessed by the Analysis of Variance (ANOVA) coupled with Duncan's multiple range test. All $p \leq 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Proximate composition and antioxidant composition of date fruit: Date fruit contained 21.57±0.06 moisture, 1.42±0.032 crude ash, 2.59±0.032 crude protein, 0.28± 0.01 crude fat, 6.3±0.21 crude fiber and 67.83±0.23 nitrogen free extract (g/100 g FW). The mean antioxidant capacity of "Medjool" DF was 1.55±0.058 mmole/100 g FW, the mean total phenolic content was 104.3±3.46 mg GAE/100 g FW and the mean value of total flavonoid content was 8.10±0.19 mg CEQ/100 g FW. Of the four phenolic compounds, only gallic acid and p-hydroxybenzoic acid were identified in the DF methanol extract. The value of gallic acid was 0.674±0.017 mg/100 g FW and the value of p-hydroxybenzoic acid was 1.59± 0.055 mg/100 g FW.

Experimentation using an animal model: Table 2 shows that there was no significant difference between experimental groups with regards to the initial body weights ($p>0.05$). The final body weights of the experimental groups were significantly higher than the baseline group ($p<0.05$) and significantly higher than the initial body weight ($p<0.01$).

Levels of oxidative stress and antioxidant capacity: Figure 1 shows TBARS levels as a marker of oxidative stress of rats among the different experimental groups. Hepatic TBARS levels expressed as nmol mL^{-1} MDA equivalents were significantly the highest in the HFD group ($p<0.01$) compared to the rest of the experimental groups. The HFD caused a percentage increase of TBARS by 59.4% from the control group. There were no significant differences between hepatic TBARS levels in the baseline, control, control+DF and HFD+DF groups ($p>0.05$).

Figure 2 shows the serum total antioxidant capacity of the rats among the different experimental groups expressed as mM Trolox equivalents. The control+DF group had the highest level of serum total antioxidant capacity than the rest of the groups but the p-value indicated a trend toward significance ($p = 0.082$). There

Table 2: Initial and final body weights, body weight gain, liver weights, accumulative food intake and food efficiency ratio of rats among experimental groups with and without df for six weeks*

Diet group	Initial body weight (g)	Final body weight (g)	Body weight gain (g)	Liver weight (g)	Accumulative food intake (g)	FER** (g/ 100 g food intake)
Baseline (n = 7)	273.9±14.3[a]	249.9±13.9[a§]	-23.9±1.4[a§]	8.2±0.32[a§]	n.d.	n.d.
Control (n = 10)	274.3±10.1[a]	356.3±10.6[b¥]	82±5.0[bc]	10.0±0.33[b]	781.9±28.8[a]	10.5±0.60[a]
Control+DF[†] (n =10)	274.5±9.7[a]	348.4±12.9[b¥]	73.7±7.0[b]	10.5±0.54[b]	771.6±24.5[a]	9.6±0.78[a]
HFD[‡](n = 10)	273.7±11.1[a]	372.7±11.1[b¥]	98.8±6.7[cd]	10.5±0.45[b]	679.7±19.1[b]	14.4±0.87[b]
HFD[‡]+DF[†] (n = 10)	273.5±11.9[a]	381.0±11.2[b¥]	107.4±9.5[d]	10.7±0.26[b]	694.8±21.2[b]	15.5±1.4[a]

DF = Date fruit; FER = Food Efficiency Ratio; n.d. = Not determined; HFD = High Fat Diet; *Data are presented as mean± SEM; Values with different superscripts within the same column are significantly different at p<0.05; **Food efficiency ratio = Body weight gain (g)/100 g food intake; [†]DF comprises 10% of diet (10 g DF/100 g diet); [‡]HFD contains 45% kcal from fat; [§]Significant at p<0.01; [¥]Significant at p<0.01 compared to initial body weight within the same row

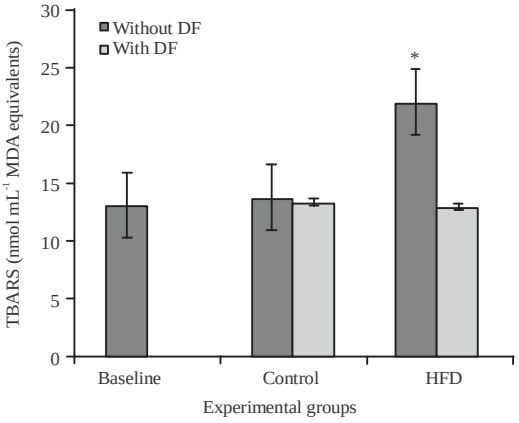

Fig. 1: Hepatic concentrations of t bars in rats among different experimental groups with and without date fruit for 6 weeks; TBARS = Thiobarbituric Acid Reactive Substances; MDA = Malondialdehyde; DF = Date Fruit; HFD = High Fat Diet; (*) Significant at p<0.01

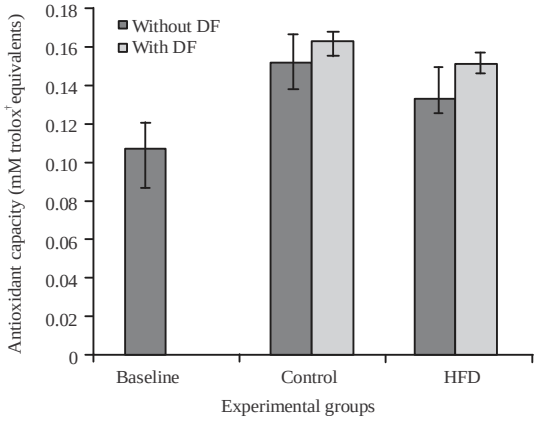

Fig. 2: Serum total antioxidant capacity of rats among different experimental groups with and without date fruit for 6 weeks; Trolox; 6-Hydroxy- 2, 5, 7, 8-tetramethyl chroman-2-carboxylic acid; DF = Date Fruit; [(†)0]Trolox is a Vitamin E analog used as a standard

were no significant differences (p>0.05) between the control+DF and control, HFD+DF, HFD and baseline groups.

Oxidative stress is the imbalance between free radicals and antioxidants. It is exacerbated in the chronic, proinflammatory state of obesity. The overproduction of ROS is cytotoxic to cell membranes leading to their oxidation and the release of oxidants such as TBARS. Fruits rich in antioxidants have antioxidative activity due to their polyphenolic content. This phenomenon was clearly demonstrated in the present study as HFD contributed to the increase of oxidative stress and "Medjool" DF with determined antioxidant capacity was portrayed as an external source of antioxidants decreasing the levels of oxidative stress in rats fed a HFD. The antioxidant properties of DF may be attributed to the total phenolic and total flavonoid content[17] as they scavenge ROS and neutralize free radicals. This illustrates their therapeutic effects such as anti-inflammatory, anti-microbial and antimutagenic activities[13].

It is assumed that HFD supplemented to rodent animal models can lead to obesity as reported by many studies[22, 27]. In this study, the body weight gain of rats in the HFD groups was significantly higher (p<0.05) than the control+DF group despite lower food intake. These variables were consistent in other publications[16, 28, 29]. Furthermore, accumulative food intake was significantly higher (p<0.05) in control groups than in HFD groups while FER was significantly lower in control groups than in HFD groups (p<0.05). These results were documented by Charradi *et al*.[29], Lee *et al*.[16] and Kim *et al*.[20].

In this study, the hepatic levels of TBARS in the HFD group were significantly the highest (p<0.01) compared to the rest of the groups. This result was in line with previous research[16, 29, 30] indicating that HFD increased hepatic TBARS levels in rats. Lipid peroxidation and antioxidant capacity are important markers of oxidative stress. Several studies have demonstrated increased lipid peroxidation products, inflammation and ROS overproduction upon HFD treatment[29, 30]. The data presented in this study emphasized that HFD is an independent risk factor for augmented lipid peroxidation in hepatic tissue as expressed by increased hepatic levels of TBARS, an oxidative stress biomarker[30]. This may be due to progressive, cumulative cell injury resulting from pressure

of the large body mass which enhances the secretion of pro-inflammatory cytokines such as TNF-α, leading to the overproduction of ROS from the tissues which react with lipids causing lipid peroxidation[30]. This results in the release of lipid peroxidation products such as MDA, hydroperoxides and hydroxyl radicals[31]. Furthermore, lipid peroxidation products instigate oxidative damage to hepatocyte mitochondria leading to the generation of more ROS and impaired respiratory chain[32].

Moreover, the findings of this research demonstrated that the TBARS levels in the HFD+DF group were significantly reduced as compared to the HFD group ($p<0.01$). This was consist with the literature Lee *et al*.[16] and Charradi *et al*.[29] and postulates the antioxidative activity of DF in rats due to their polyphenolic content. These findings can be explained by the DF's high content of antioxidant substances such as phenolics and flavonoids which have been proven to scavenge free radicals such as peroxide, hydroperoxide or lipid peroxl and thus protect against oxidative stress[17]. Abundant literature has proved the crucial role in utilizing fruits rich in antioxidants to improve the oxidative damage induced by a HFD[16, 29]. Our results are in accordance with these results and further strengthen the importance of the active compounds present in DF specifically to ameliorate oxidative stress.

Furthermore, the findings in the present study showed that the control+DF group had the highest antioxidant capacity among the groups while the p-value only showed a trend toward significance ($p = 0.082$). This was consistent with the findings of Ramiro-Puig *et al*.[33] who reported that total antioxidant capacity in rat plasma did not significantly differ in rats fed diets supplemented with 10% cocoa compared to control. The decrease of total antioxidant capacity in the serum of rats may be attributed to the short half-lives of flavonoids as blood was collected after 14 h fast. Therefore, the time gap between DF intake and blood collection could be a possible reason for the insignificant differences in total antioxidant capacity among groups[33]. In addition, Ramiro-Puig *et al*.[33] determined that there was a significant increase in total antioxidant capacity in hepatic tissues as opposed to plasma which shows that antioxidant accumulation was increased in specific target tissues such as the liver. There are various assays used to measure antioxidant capacity in biological samples and therefore the use of a single measurement may not truly reveal the overall antioxidant status[34].

CONCLUSION

The findings of the present study demonstrated that "Medjool" variety of DF at the Tamr stage are one of the highest in antioxidative compounds such as phenolics and flavonoids contributing to its total antioxidant capacity. "Medjool" DF incorporation in the diet has proven to have antioxidant effects by improving the hepatic levels of TBARS, a biomarker of lipid peroxidation and tending to increase total antioxidant capacity in rats. Dietary intake of "Medjool" variety of DF could have a potential role in ameliorating oxidative damage induced by obesity through the reduction of free radicals and ROS to maintain the oxidant-antioxidant balance and possibly reduce the risk of obesity co-morbidities.

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