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The Effect of Date Palm Fruit (*Phoenix dactylifera* L.) on the Enzyme Glutathione-S-Transferase Activity in Sprague-Dawley Rats

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Abstract: This research was performed to study the effect of date palm fruit (*Phoenix dactylifera* L.) on the hepatic enzyme system glutathione-S-transferase (GST) in rats with 7, 12-dimethylbenz (alpha) anthracene (DMBA)-induced mammary cancer. The effect of feeding the fruit was compared to that of feeding raw soybean seeds and injection with 2 drugs (preventive and curative against mammary cancer) i.e., tamoxifien and 17-beta-estradiol. Eighty three female weanling Sprague-Dawley rats were randomly distributed into 8 groups. Each group received one of the dietary or drug treatments. One of the groups was injected with sesame oil which is the carcinogen vehicle, to serve as a negative/DMBA control group. Another group of rats that received neither drug nor special dietary treatment served as a positive/DMBA control group. The experiment lasted for 26 weeks. At the end of the feeding period, all animals were killed, livers were removed, prepared and analyzed for their protein content and GST enzyme activity. Livers of the rats that were injected with sesame oil vehicle showed the highest enzyme activity. The rats which were fed the date palm fruit tended to (p = 0.079) reduce the enzyme activity. It is concluded that the date palm fruit at the 2 maturity stages may possess antioxidant activity that is reflected positively in the prevention of DMBA-induced mammary cancer.

Key words: Date palm fruit, Glutathione-S-transferase (GST), DMBA, Mammary cancer

INTRODUCTION

Mammary cancer is a neoplastic change in the epithelial cells of the mammary tissue (Guzman *et al.*, 1999). The use of 7, 12-dimethylbenz (alpha) anthracene (DMBA) to induce mammary cancer in rodents, is one of the most two commonly used models to study mammary cancer (Guzman *et al.*, 1999).

When entering the body, xenobiotics either directly produce Reactive Oxygen Species (ROS) (Talalay *et al.*, 1997) or induce indirectly their production. The presence of these molecules in the cells is associated with imbalance in the oxidative status of the cells. The imbalance of cell oxidative status is associated with the development of "oxidative stress" diseases such as cancer. Additionally, ROS promote cell proliferation (Townsend *et al.*, 2003). The exposure of the body to various chemicals obligates its cells to get rid of them. For this purpose, the body is equipped with various mechanisms to detoxify toxic chemicals into more water-soluble compounds where the body can excrete them safely (Habig *et al.*, 1974).

Xenobiotics are metabolized in the body by one or both of two pathways: Phase I (oxidative functionalization reactions) and Phase II (biosynthetic reactions). In Phase I reactions, the reactive functional group of the xenobiotic is removed. Phase II enzymes connect these functional groups to endogenous molecules such as

glutathione (Talalay *et al.*, 1997; Roman and Masters, 2006). Glutathione-S-transferases are group of 6 Phase II detoxification enzymes present mainly in the cell cytosol. These enzymes catalyze the conjugation of reactive electrophilic group of xenobiotics to the reduced Glutathione Molecule (GSH) (Talalay *et al.*, 1997). Additionally, GSTs regulate the cell survival-apoptosis balance (Townsend *et al.*, 2003).

The GST enzyme system conjugates the electrophilic reactive groups (R) of xenobiotics with the reduced glutathione (GSH) molecule to convert the reactive functional group of xenobiotics into more hydrophilic compounds that can be easily excreted according to the following equation (Habig *et al.*, 1974):

$$GSH + R \rightarrow GS - R$$
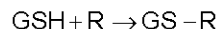
It has been shown by Roman and Masters (2006) that decreased Phase II enzyme activity is associated with inability to detoxify carcinogens and increased susceptibility to various types of cancers.

Several studies examined the antioxidant power of various extracts of the date palm fruit (Qusti *et al.*, 2010; Khanavi *et al.*, 2009). According to the available literature, no study had examined the antioxidant effect of the whole date palm fruit at the *in vivo* level. Thus, this study aimed at testing the antioxidant effect of date palm

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fruit (*Phoenix dactylifera* L.) by testing the enzyme system glutathione-S-transferase (GST) in the livers of rats with DMBA-induced mammary cancer.

MATERIALS AND METHODS

Date palm fruit at the growing stage "Rutab" was washed with tap water and air-dried over cotton cloths. Due to the high moisture content of "Rutab", it was decided to freeze-dry the fruit. Freeze-drying was performed in the Telstar Crvodos ® laboratory freeze-dryer (Spain) at 2.5 mbar pressure at -70 to -80°C. Date palm fruit at the two maturity stages ("Rutab" and "Tamr") was pitted and passed through a food mincing machine (Kenwood®, UK) with an opening size of 4 mm until it became smooth paste. Soybean seeds were cleaned from stones and grinded into powder using a blender (Waring Commercial blender®, USA).

Test meals which were introduced to the animals were prepared to be isocaloric and isonitrogenous according to the guidelines of the American Institute of Nutrition-1993 for growing animals (AIN-93G) recommended by Reeves, 1997 (Table 1) with some modifications as follows:

• Replacement of soybean oil with an oil mixture composed of canola oil and sunflower oil in a ratio of 0.77:1.0
• Powdered soybean seeds were added at a level of 30% of the diet (AIN-93G+Soybean) weight to provide 50% of the protein requirements according to the recommendations of Constantinou *et al.* (2001). The rest of the protein requirements were provided by casein. For comparison purposes: freeze-dried "Rutab" and "Tamr" were added at the same weight. The proximate composition of the plants was taken into consideration and the experimental diet contents of carbohydrates, protein and fiber were completed by starch, casein and cellulose according to the recommendations of Reeves (1997). Test meals were of four types: AIN-93G, AIN-93G containing soybean seeds (AIN-93G+Soybean), AIN-93G containing freeze dried "Rutab" (AIN-93G+"Rutab") and AIN-93G containing "Tamr" (AIN-93G+"Tamr")

Animal experimentation
Animal feeding: Eighty three female weanling Sprague-Dawley (SD) rats (37.6-40.6 g body weight) were weighed to the nearest 0.1 g and distributed into eight groups according to their initial body weights. Since then -and throughout the experiment- animals were placed individually in metabolic cages in the Animal Unit, Department of Nutrition and Food Technology, The University of Jordan at controlled temperature (25±2°C), humidity (50±5%) and 12 h light-dark cycle. Animals were fed the AIN93-G meals for a week of acclimatization. After that, each group of animals received one of the four test meals and drank tap water for 26 weeks.

The administration of drugs and carcinogen: At the age of 5 weeks, a group of animals (fed AIN-93G meals) was injected once per week subcutaneously with 2.5 mg TAM (Sigma, purity = 95%) (dissolved in ethanol and suspended in sesame oil)/kg body weight/day/4 weeks Heffelfinger *et al.* (2003). For comparison purposes, a group of animals (fed AIN-93G meals) was injected with TAM vehicle (sesame oil) without TAM once per week for 4 weeks. At the age of 7 weeks, 7 groups of animals (the two that received the TAM and TAM vehicle, two fed AIN-93G, one fed AIN-93G+Soybean, one that received AIN-93G+"Rutab" and one that received AIN-93G+"Tamr") were orally gavaged with 15 mg DMBA/kg/day once (Teller *et al.*, 1977). The DMBA was of Sigma, purity = 95%; it was dissolved in acetone, suspended in sesame oil and acetone was then evaporated by liquid nitrogen gently). For comparison purposes, one of the animal groups (fed AIN-93G meals) was orally gavaged with DMBA vehicle (sesame oil) to serve as the negative control group (negative/DMBA). After the development of at least one palpable structure with = 1 cm in one of 2 measured perpendicular axis, a group of animals (fed AIN-93G meals) were injected daily subcutaneously at the scruff region with 1.6 mg estradiol hemihydrates (Sigma, purity = 95%, dissolved in ethanol and suspended in sesame oil) /kg/day/four weeks; a procedure recommended by Teller *et al.* (1977) to regress the tumor size. For comparison purposes, the same group of animals (which was injected with TAM

Table 1: Formulation of the test diets used in the animal feeding experiments

| Diet | Component (g or mg/kg diet) | | | | | | | | | | |
	Plant	CS	Casein[1]	Sucrose	OM[2]	Fiber[1]	MM[3]	VM[4]	L-Cystine	CB[5]	TBHQ[6] (mg)
AIN-93G	0	529.5	200	100	70	50	35	10	3.0	2.5	14
AIN-93G+Soybean	300	512.5	100	0	16	21	35	10	3.0	2.5	14
AIN-93G+"Rutab"	300	359.5	191	0	69	30	35	10	3.0	2.5	14
AIN-93G+"Tamr"	300	344.2	194	0	69	42.3	35	10	3.0	2.5	14

[1]Casein (> 85% protein) and fiber (alpha-cellulose) were purchased from LabDiet ®
[2]Oil mixture composed of 56.4: 43.6 sunflower: canola oil
[3,4]Vitamin and mineral mixtures were prepared according to Reeves *et al.* (1997)
[5]Choline bitartarate (41.1% choline)
[6]TBHQ = Tert-butylhydroquinone
CS: Corn starch, OM: Oil mixture, MM: Mineral mix, VM: Vitamin mix, CB: Choline bitartarate

vehicle) was injected with E_2 vehicle (sesame oil) without E_2 daily for 4 weeks. A group of animals (fed AIN-93G) was left without injection of any drug (neither TAM nor E_2) to serve as the positive control group (positive/DMBA control group). Three weeks after the carcinogen administration, animals were palpated twice per week to record the appearance of any structure (s) by day and date according to Huggins *et al.* (1964). Four weeks after ending estradiol injection, all animals were fasted for 16 hours and killed by chloroform anesthesia. Animal livers were removed, weighed (Sartorius®, USA) and stored in the deep freezer at -80°C until analyzed for their protein content and glutathione-S-transferase (GST) activity.

At the end of the feeding period, all animals were killed, their livers were removed and prepared for analysis of the activity of hepatic GST.

Analysis of livers

Analysis of hepatic GST activity: Livers were thawed gradually, minced with a blade and 3 milliliters of phosphate buffered saline (PH = 7.0-7.2) were added to each gram of liver. Buffer-liver mixture was homogenized by a portable homogenizer (IKA® T18 basic Ultra-turrax, Germany) for less than 1 minute while the beaker was submerged in ice. Liver homogenates were centrifuged at 10, 000 xg at 4°C/15 minutes (Hermle labor technik GmBh®, Germany), a sample was kept (without centrifugation) for liver protein content analysis. Hepatic GST activity was assayed according to Habig *et al.* (1974) using a special kit for the enzyme analysis (Cayman, catalog No. 703302, USA). The enzyme activity was assayed by measuring the conjugation of 1-Chloro-2, 4-dinitrobenzene (CDNB) with reduced glutathione. The conjugation was accompanied by an increase in absorbance at 340 nm. Absorbance change was measured immediately for 10 minutes using UV/Visible spectrophotometer (Perkin Elmer Lambda 25, UK) in 1 cm quartz cuvettes. Hepatic GST activity was expressed per mg protein content of the liver.

Analysis of hepatic protein content: Hepatic protein content was analyzed according to the method of Bradford (1976). This method involves the binding of Coomassie Brilliant Blue to the liver protein. This binding was accompanied with an increase in absorbance (after two minutes of reaction) at 595 nm in 3 cm cuvettes measured by UV/Visible spectrophotometer (Perkin Elmer Lambda 25, UK). Absorbance was measured for standard protein solutions of bovine serum albumin (BSA, Sigma, purity = 95%) and sample absorbance reading was used to extrapolate the concentration of hepatic protein content.

Statistical analysis: The statistical analysis was carried out using the statistical analysis system (SAS). Food intake and liver data were analyzed by one way analysis of variance (ANOVA) procedure using Fisher's (F-test) protected least significant difference (LSD) to detect significant differences between the treatment means.

RESULTS

Animal experimentation

Food intake: The highest food intakes (Table 2) were found in both the AIN-93G+"Tamr" (2497.79±85.21) and AIN-93G+"Rutab" (2457.49±95.27) groups, followed by the AIN-93G+E_2 (2261.24±77.79) and the positive/DMBA control group (2232.62±85.21). The remaining groups had lower food intake with no significant differences ($P>0.05$) among them.

The activity of hepatic GST: Results of the hepatic GST activity show that there were no significant differences ($p = 0.079$) among the rat groups (Table 3). Nonetheless, the groups which were fed AIN-93G+Sesame oil, AIN-93G+Soybean and AIN-93G+"Rutab" tended to express high values for hepatic GST activity (2418.90±483.0, 1711.4±483.0 and 1650.4±577.3 respectively). The AIN-93G+"Tamr", AIN-93G+TAM and the AIN-93G+E_2 tended to express moderate values for the enzyme activity (1139.2±483.0, 966.00±423.6 and 959.50±460.5 respectively). Whereas the negative/DMBA and the positive/DMBA control had the lowest GST activity values (283.10±183.0 and 693.00±483.0 respectively).

DISCUSSION

Animal experimentation: Food intake can be explained with reference to many factors such as: animal health, physiological status, food palatability, familiarity with the food and other external factors (Hubert *et al.*, 2000). The high sugar content of the date palm fruit may be

Table 2: Food intake of the experimental groups throughout the experiment[1]

Group (n)[2]	AFI (g)
Negative/DMBA control group (10)	2139.99±85.21[c]
AIN-93G+Sesame oil (10)	2147.05±85.21[c]
AIN-93G+TAM (13)	2072.78±74.74[c]
Positive/DMBA control group (10)	2232.62±85.21[bc]
AIN-93G+E_2 (12)	2261.24±77.79[bc]
AIN-93G+Soybean (10)	2198.56±85.21[c]
AIN-93G+"Rutab" (10)	2457.49±95.27[ab]
AIN-93G+"Tamr" (10)	2497.79±85.21[a]

[1]Values are expressed as mean±SEM, values with different letters within a column differ significantly at ($p<0.05$) using Fischer's protected LSD test
[2]AIN-93G=diets recommended by the American Institute of Nutrition-1993 for growing rats, n=number of rats used within each experimental group, TAM= tamoxifen, E2=17-beta-estradiol, DMBA=7,12-dimethylbenza (alpha) anthracene, g = gram, AFI: Accumulative food intake

Table 3: Liver weight, liver protein content, and hepatic GST activity of the experimental groups

Group (n)[2]	Liver weight (g)	Liver protein content (mg)	Hepatic GST activity (nmol/minute/mg protein)
Negative/DMBA control (10)	6.54±0.37[a]	1278.01±300.90[a]	283.10±183.0[a]
AIN-93G+Sesame oil (10)	6.79±0.37[a]	1154.54±300.90[a]	2418.90±483.0[a]
AIN-93G+TAM (13)	5.95±0.32[a]	1444.68±263.90[a]	966.00±423.6[a]
Positive/DMBA control (10)	7.07±0.37[a]	1693.38±300.90[a]	693.00±483.0[a]
AIN-93G+E$_2$ (12)	6.70±0.34[a]	1394.62±274.68[a]	959.50±460.5[a]
AIN-93G+Soybean (10)	6.51±0.37[a]	1771.98±300.90[a]	1711.4±483.0[a]
AIN-93G+"Rutab" (8)	6.77±0.42[a]	934.16±336.41[a]	1650.4±577.3[a]
AIN-93G+"Tamr" (10)	7.00±0.37[a]	1386.65±300.90[a]	1139.2±483.0[a]

[1]Values are expressed as mean ± SEM,
Values without different letter within a column differ significantly at ($p < 0.05$) using Fischer's protected LSD test
[2]Abbreviations are as follows: AIN-93G= diets recommended by the American Institute of Nutrition-1993 for growing rats, n= number of rats used within each experimental group, TAM= tamoxifen, E$_2$=17-beta-estradiol, DMBA=7,12-dimethylbenza(á)anthracene.

responsible for the fruit high palatability. This was expressed by the highest intake observed in the groups which were fed AIN-93G+"Tamr" and AIN-93G+"Rutab".It was found in this work that the groups AIN-93G+Sesame oil and AIN-93G+TAM tended to have lower food intakes than that of AIN-93G+Soybean. Injection (either with the drugs or with their vehicle i.e., sesame oil) is a factor to which animals were exposed. One of the observations noted in this research was that after injection, animals expressed reduced activity and food intake after injection. Thus, it seems that the injection is invasive for animals and a negative effect of multiple injections on animals' food intake must not be ruled out. Rats which were injected with TAM showed the lowest food intake among the rat groups. This is consistent with the result of other authors (Lope'z *et al.*, 2006). To prevent/cure mammary cancer, tamoxifen acts as an antiestrogen; TAM is thought to reduce food intake by reducing hepatic fatty acid synthase (FAS)-mRNA expression and accumulating malonyl CoA in the hypothalamus instead of using it in fatty acid elongation (Lope'z *et al.*, 2006). The relatively moderate-low (p<0.05) food intake of the AIN93G+Soybean diet may be partially explained with reference to the presence of dietary fiber. Dietary fiber holds water and induces filling effect in the stomach (Guillon *et al.*, 2000). The relatively moderate-low food intake expressed in the positive control group can be explained with reference to the animal health status. This group of animals was administered with DMBA without any curative or preventive drug; additionally, they were not fed any special diet. Cancer is known to induce anorexia (Medina, 2001) in humans and animals; thus, the low food intake expressed by this group would have been expected.

The modulation of Phase I and Phase II enzyme systems by natural compounds is known to be protective against DMBA-induced cancer initiation. It has been known that the modulation of Phase II enzymes, especially GST, is closely correlated with the reduction in the number of induced tumors in rodents (Krajka-Kuz'niak *et al.*, 2011).

The activity of hepatic GST: The GSTs are group of enzymes that are known to be important in drug and carcinogen metabolism (Haibeg *et al.*, 1974). The activity of hepatic GST depends on many factors such as: the presence of xenobiotics (Haibeg *et al.*, 1974) and diet (Hubert *et al.*, 2000), yet is affected by individual variations (Constantinou *et al.*, 2001). The compounds (whether natural or synthetic) that are capable of modulating phase II enzymes in carcinogen metabolism interfere with the process of carcinogenesis (Habig *et al.*, 1974).

Consistent with the previous studies (Clifton *et al.*, 1975), the administration of DMBA in this research tended to enhance the activity of hepatic GST (p = 0.079). All the rat groups which were administered the carcinogen DMBA tended to express higher hepatic GST activity because the simultaneous detoxification of the most potent DMBA metabolite (i.e., 3, 4-dihydrodiol-1, 2-epoxide) occurs by phase II enzymes (Clifton *et al.*, 1975). The AIN-93G+Sesame oil group did not receive special dietary or drug treatment; it is probable that this was the reason for the relatively high GST activity values of this group. The inhibition of GST activity of the AIN-93G+TAM is consistent with the result of Nuwaysir *et al.* (1996). Additionally, feeding phytoestrogens such as genestein (Guillon *et al.*, 2000), antioxidants, polyphenols (Wiegand *et al.*, 2009), or choline (Rashwan, 2012) were reported to inhibit hepatic GST activity.

The presence of functional antioxidant compounds (such as Se, polyphenols, phytohormones and carotenoids) in the soybean and date palm fruit in the "Rutab" and "Tamr" stages might be responsible for lowering the values of hepatic GST activity and are thought to be responsible for inhibition of DMBA-induced mammary cancer (Sohn *et al.*, 1999).

These results suggest also, that the anti-cancer effect of date palm fruit that was obtained in this research might be due to either mechanism (s) other than the antioxidant mechanism or due to enzyme systems other than hepatic GST. Other antioxidant enzyme system (s) suggested include superoxide dismutase, catalase and

glutathione peroxidase. Saafi *et al.* (2011) found a significant increase in the hepatic activity of these enzymes after treatment of adult male dimethoate-reated Wistar rats with an aqueous extract from date palm fruit (4ml/kg body weight) for 2 months. Similarly, feeding a mixture of date palm fruit and camel milk to adult male Swiss Albino rats (administered lead actate to induce lipid perooxidation) for 7 weeks enhanced the activity of serum GST (Decedas *et al.*, 1994). Data of the hepatic GST activity suggest-although do not prove- that the date palm fruit at the two maturity stages "Rutab" and "Tamr" possess antioxidant activity. This result is in agreement with the results of the tumor incidence results obtained in our laboratory (Al-Sayyed *et al.*, 2013).

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