

The effect of date palm fruit (*Phoenix dactylifera* L.) on 7, 12-dimethylbenz (α) anthracene (DMBA)-induced mammary cancer in rats

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Abstract

This research was performed to study the effect of date palm fruit (*Phoenix dactylifera* L.) on the 7,12-dimethylbenz (α) anthracene-induced mammary cancer in rats. The effect of feeding the fruit at two maturity stages ("Rutab" and "Tamr") was compared to that of feeding raw soybean seeds and injection with two drugs, preventive and curative against mammary cancer, i.e., tamoxifen and 17- β -estradiol respectively. For comparison purposes, another group of rats was injected with sesame oil. Eighty three female weanling Sprague-Dawley rats were randomly distributed into the treatment groups. Each group received one of the dietary or drug treatments. Another group of rats that received neither drug nor special dietary treatment served as a positive/carcinogen control group. Still another group was injected with sesame oil, which is the carcinogen vehicle at the time of injecting the carcinogen, to serve as a negative/carcinogen control group. Three weeks after the carcinogen administration, animals were palpated twice per week to record the appearance of any lesion. At the end of the 26-week feeding period, all animals were killed. Palpable lesions, mammary tissue, and associated lymph nodes were removed, fixed, and prepared for histopathological examination. The diets that contained the date palm fruit reduced significantly ($P<0.05$) the incidence rate of mammary cancer, palpable tumour multiplicity, tumour size and weight compared to the positive/DMBA control group. In conclusion, the date palm fruit (*Phoenix dactylifera* L.) protected against DMBA-induced mammary cancer in the initiation, promotion, and progression stages of DMBA-induced mammary carcinogenesis. The effect was comparable with that of the two drugs (tamoxifen and 17- β -estradiol). Further research is required to elucidate the possible mechanisms that might have contributed to the preventive effect of this fruit against mammary cancer.

Keywords: Date palm fruit; "Rutab"; "Tamr"; 7, 12-dimethylebenza (α) anthracene; mammary cancer

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Introduction

Mammary cancer is a neoplastic change in the epithelial cells of the mammary tissue. The lesion occurs commonly in the epithelium of the mammary ducts (Guzman et al., 1999). Mammary cancer caused 460000 deaths in 2008 worldwide (Jemal et al., 2010). In 2009, breast cancer was ranked as number one among the ten most common cancers in Jordanian females (MoH, 2009).

It has been estimated that the environmental factors can contribute to the development of 90-95% of all

cancers and only 5-10% of cancers are caused by genetic defects (Anand et al., 2008). This high contribution of environmental factors led health care providers to focus on searching the food constituents that may act against cancer.

The principle of cancer prevention by dietary modification is the use of biological activity of certain compounds present in the food that can modify one or more of the steps involved in the process of carcinogenesis (Tsuda et al., 2004).

The date palm tree (*Phoenix dactylifera* L.) is a perennial monocotyledon plant of the family *Palmae*

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(Vayalil, 2012). Date palm fruit is a rich source of sugars, minerals, and contains other functional constituents such as antioxidant vitamins, flavonoids, polyphenols, phytoestrogens (Vayalil, 2012), and phytemelatonin (Al-Qarawi et al., 2003). Several studies examined the medical effect of date palm fruit on the stimulation of immunity (Pereira et al., 2011) and against cancer (Vayalil, 2002; Ishrud and Kennedy, 2005). The use of 7,12-dimethylbenz (α) anthracene (DMBA) to induce mammary cancer in rodents, is one of the two most commonly used models to study mammary cancer. The tumours produced are carcinomas of ductal origin (Guzman et al., 1999).

The aim of this study was to test the effect of date palm fruit of (Barhi) type on the DMBA-induced mammary cancer using the rat model.

Materials and Methods

Preparation of the plants and diets used in this research

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).

Date palm fruit at the edible maturity stages "Rutab" (incompletely mature) and "Tamr" (mature fruit) was incorporated into rat diets. Soybean seeds were also incorporated into the rat diets as soybean seeds were known to be protective against DMBA-induced mammary cancer (Hakkak et al., 2000). All of the 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) (Table 1) recommended by Reeves (1997) with some modifications as follows:

A: Replacement of soybean oil with an oil mixture composed of canola oil and sunflower oil in a ratio of 0.77:1.0.

B: 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 fibre 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

Eighty three female weanling healthy Sprague-Dawley (SD) rats weighing 37.6-40.6 g body weight were used in this research. The animals were placed at a controlled temperature of 25±2°C, humidity (50±5%), and 12-hour light-dark cycle in the Animal Unit/Department of Nutrition and Food Technology/Faculty of Agriculture/The University of Jordan.

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 tamoxifen (TAM) (Sigma, purity = 95%) (Dissolved in ethanol and suspended in sesame oil)/kg body weight/day for 4 weeks according to 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 (Sigma, purity= 95%) 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 lesion (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 (E₂) (Sigma, purity = 95%, dissolved in ethanol and suspended in sesame oil) /kg/day/four weeks). Three weeks after the carcinogen administration, animals were palpated twice per week (till the end of the feeding experiment) to record the appearance of any lesion (s) by day and date (Huggins et al., 1964). Four weeks after ending estradiol injection, all animals were fasted for 16 hours and killed by chloroform anaesthesia. Mammary tissues (cervical, thoracic, abdominal, and inguinal) and associated lymph nodes, and palpable lesions were removed, their sizes were measured grossly in three dimensions by a digital calliper (TRESNA[®], USA), weighed (Sartorius[®], USA), and submerged and stored in 10% saline formalin for fixation until histopathological examination.

Table 1: Formulation of the test diets used in the animal feeding experiments (Al-Sayyed et al., 2013)

Diet (g/kg diet)	Plant starch	Corn starch	Casein ¹	Sucrose	Oil mixture ²	Fiber ¹	Mineral mix ³	Vitamin mix ⁴	L-Cystine	Choline bitartrate ⁵	TBHQ ⁶ (mg)
AIN-93G ⁷	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

¹Casein (> 85% protein) and fiber (α -cellulose) were purchased from LabDiet[®]; ²Oil mixture composed of 56.4: 43.6 sunflower: canola oil; ³, ⁴Vitamin and mineral mixtures were prepared according to Reeves et al. (1997); ⁵Choline bitartrate (41.1% choline); ⁶TBHQ=Tert-butylhydroquinone; ⁷This diet was used in the first five treatments as shown in other tables; ⁷ This diet was used in the first five treatments as shown in other tables

Histopathological examination of tissues

Tissues were prepared according to Russo and Russo (2000) and examined for confirmation of the presence of tumour tissue under electron microscope (OLYMPUS, USA) (100 X power) by a histopathologist.

Statistical analysis

The statistical analysis was carried out using the statistical analysis system (SAS). Data of tumour latency and tumour size 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. The data of tumour incidence was analyzed using the proposed likelihood-ratio testing according to Kokoska et al. (1993). The tumour multiplicities were analyzed by Chi square test (χ^2) for equal proportions. Fischer's exact test was performed to compare each pair of treatments only when statistical significance was detected.

Results

The incidence of mammary cancer among the experimental groups

Fig. 1 shows the histology of mammary tissue with invasive mammary ductal adenocarcinoma compared to normal mammary tissue. Results of histopathological examination of the tumours showed that the tumours were of the invasive mammary ductal adenocarcinoma (IMDAC) type. The results of tumour incidence rates are shown in Table 2. No tumours were found in the negative carcinogen control and AIN-93G+soybean groups. The highest incidence rate of IMDAC was in the AIN-93G+TAM group. Lower incidence rates than the positive/DMBA and the AIN-93G+TAM were found in the AIN-93G+"Rutab" and AIN-93G+"Tamr" groups.

Tumour latency

In this research, the PIMDAC appeared between weeks 6-23 after DMBA administration. The earliest palpable tumours appeared in the AIN-93G+Sesame oil, the positive/DMBA control groups, and the AIN-93G+E₂ (Table 3). Later palpable tumours were detected in the

AIN-93G+TAM, AIN-93G+"Rutab" group and AIN-93G+"Tamr" group.

The PIMDAC multiplicity, size, and weight

The highest tumour multiplicity (Table 4) was in the AIN-93G+Sesame oil and the AIN-93G+TAM, and AIN-93G+E₂. Feeding the AIN-93G+"Rutab" and AIN-93G+"Tamr" diets tended to reduce tumour multiplicity. The largest ($p<0.05$) maximum tumour diameters (mm) (Table 4) were expressed in the AIN-93G+Sesame oil and the positive/DMBA control groups. Smaller tumour diameters were shown in the AIN-93G+"Rutab" followed by the AIN-93G+E₂, AIN-93G+TAM, and AIN-93G+"Tamr" groups. Tumour weight almost followed the same pattern (Table 4). The heaviest tumours (mg) appeared in the positive/DMBA control group and the AIN-93G+Sesame oil group. Lighter tumours were expressed in the AIN-93G+"Tamr", AIN-93G+E₂, AIN-93G+TAM, and AIN-93G+"Rutab" groups.

Discussion

The incidence of mammary cancer among the experimental groups

The tumours induced in this research were of the typical type of mammary tumours induced by DMBA (Guzmn et al., 1999). The initiation of DMBA-induced mammary cancer occurs when the most potent DMBA metabolite (3,4-dihydrodiol-1,2-epoxide) irreversibly binds to deoxyadenosine and deoxyguanosine residues of the DNA forming DNA-DMBA adducts (Singletary and Milner, 1987). The DMBA-DNA adduct formation is positively correlated with tumour incidence (Higer, 1961). It is not easy in the present study to explain the significance of lower food intake in the AIN-93G+Soybean rats. Soybean is a rich source of many functional compounds such as dietary fibre. The date palm fruit at the two maturity stages is also a good source of dietary fibre, though the type of dietary fibre is different in both diets. It is possible that the cancer preventive effect in soy rats is attributed to, at least partially, the dietary fibre. The dietary fibre content of all experimental diets was similar. The difference was in the type of dietary fibre. The AIN-93G diets contained α -

Table 2: The incidence ¹ of IMDAC, PIMDAC, and NPIMDAC among the experimental groups²

Group (n) ²	Incidence of		
	IMDAC	PIMDAC	NPIMDAC
AIN-93G /DMBA negative control ^{2,3} (10)	0 (0%) ^{def}	0 (0%) ^a	0 (0%) ^a
AIN-93G/drug control ³ (10)	4 (40%) ^{ac}	3 (30%) ^a	1 (10.0%) ^a
AIN-93G+TAM (13)	6 (46.2%) ^{abc}	5 (38.46%) ^a	1 (7.7%) ^a
AIN-93G/DMBA positive control (10)	3 (30%) ^{bd}	1 (10%) ^a	2 (20%) ^a
AIN-93G+E ₂ (12)	3 (25%) ^{cde}	3 (25%) ^a	0 (0%) ^a
AIN-93G+Soybean (10)	0 (0%) ^{def}	0 (0%) ^a	0 (0%) ^a
AIN-93G+"Rutab" ³ (8)	1 (12.5%) ^{cde}	1 (12.5%) ^a	0 (0%) ^a
AIN-93G+"Tamr" ³ (10)	2 (20%) ^{ce}	1 (10%) ^a	1 (10%) ^a

¹Data is expressed as number of rats (% of rats), values with different letters within a column differ significantly at (p<0.05) using Fischer's exact test; ²Abbreviations are as follows: AIN-93G= diets recommended by the American Institute of Nutrition-1993 for growing rats, IMDAC= invasive mammary ductal adenocarcinoma, NPIMDAC= non palpable invasive mammary ductal adenocarcinoma, PIMDAC= palpable invasive mammary ductal adenocarcinoma, n=number of rats used within each experimental group, TAM= tamoxifen, E₂=17-β-estradiol, DMBA=7,12-dimethylbenz(α)anthracene; ³Sesame oil was used as a carrier for both the carcinogen (DMBA) as well as for the drugs (tamoxifen and 17-β-estradiol)

Table 3: Palpable mammary tumour multiplicity¹, site and latency³

Group (n) ⁴	Multiplicity of PIMDAC (Total number of PIMDAC/group) ¹	Site of PIMDAC development (mammary gland) ²	Latency of PIMDAC (day after DMBA administration) ^{2,3}
AIN-93G /DMBA negative control ^{4,5} (10)	0 ^c	No tumours detected	No tumours detected
AIN-93G/drug control ⁵ (10)	6 ^{ab}	2 thoracic 2 inguinal 2 abdominal	42.6±13.7 ^b
AIN-93G+TAM (13)	5 ^{ab}	1 thoracic 2 cervical 1 abdominal 1 inguinal	70.3±11.9 ^b
Positive/DMBA control group (10)	2 ^{bc}	2 inguinal	49.0±23.8 ^b
AIN-93G+E ₂ (12)	4 ^{ab}	2 thoracic 1 abdominal 1 inguinal	51.2±13.7 ^b
AIN-93+Soybean (10)	0 ^c	No tumours detected	No tumours detected
AIN-93G+"Rutab" ³ (8)	1 ^{bc}	Inguinal	162.0±23.8 ^a
AIN-93G+"Tamr" ³ (10)	1 ^{bc}	Inguinal	90.0±23.8 ^{ab}

¹Values are expressed as total number of PMDAC/group, values with different letters within a column differ significantly at (p<0.05) using Fischer's exact test; ²Expressed as number of tumours developed in the specified mammary gland; ³Expressed as mean day (after DMBA administration) ± SEM, values with different letters within a column differ significantly at (p<0.05) using Fischer's protected LSD test; ⁴Abbreviations are as follows: AIN-93G=diets recommended by the American Institute of Nutrition-1993 for growing rats, PIMDAC= palpable invasive mammary ductal adenocarcinoma, n= number of rats used within each experimental group, TAM=tamoxifen, E₂=17-β-estradiol, DMBA=7, 12-dimethylbenz (α) anthracene; ⁵Sesame oil was used as a carrier for both the carcinogen (DMBA) as well as for the drugs (tamoxifen and 17-β-estradiol)

Table 4: Average largest diameter and average weight of the palpable tumours among experimental groups¹

Group (n) ²	Largest Diameter of the Palpable Tumours (mm)	Average Tumour Weight (mg)
AIN-93G /DMBA negative control ^{2,3} (10)	no tumours detected	no tumours detected
AIN-93G/drug control ³ (10)	10.56±0.91 ^a	2610±410 ^a
AIN-93G+TAM (13)	2.87±0.71 ^c	70±320 ^b
AIN-93G/DMBA positive control (10)	9.50±1.57 ^{ab}	4220±710 ^a
AIN-93G+E ₂ (12)	3.52±0.91 ^c	180±510 ^b
AIN-93G+Soybean (10)	no tumours detected	no tumours detected
AIN-93G+"Rutab" ³ (10)	1.33±1.57 ^c	60±710 ^b
AIN-93G+"Tamr" ³ (8)	4.51±1.57 ^{bc}	200±710 ^b

¹Values are expressed as mean (of tumour bearing rats) ± SEM, values with different letters within a column differ significantly at (p<0.05) using protected Fischer LSD-test; ²Abbreviations 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, E₂= 17-β-estradiol, DMBA= 7,12-dimethylbenz (α) anthracene; ³Sesame oil was used as a carrier for both the carcinogen (DMBA) as well as for the drugs (tamoxifen and 17-β-estradiol)

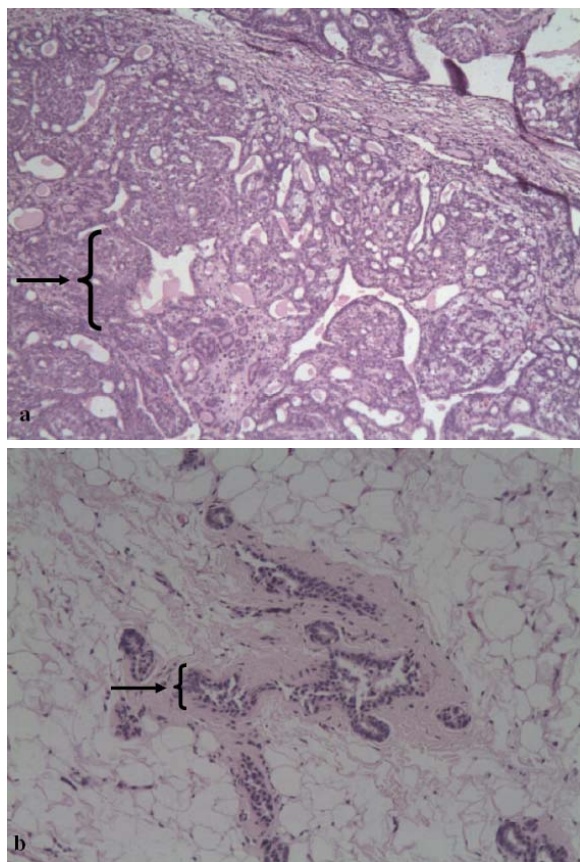


Fig 1: Histology of a. mammary tissue with invasive mammary ductal adenocarcinoma compared to b. normal mammary tissue (H & E Stain, magnification power: 100X)

cellulose as the fiber source. Also, cellulose is the main component of dietary date palm fruit in addition to hemicelluloses, and lignin (Al-Qarawi et al., 2003). However, the types of dietary fiber present in soybeans are pectins, galactomannans, and arabinogalactans (Pereira et al., 2011). The proposed effect of dietary fibre in reducing the incidence rate in this research is consistent with that reported by Pereira et al. (2011). The mechanisms by which dietary fibres intake reduce the risk of mammary cancer has been intensively studied (Vayalil, 2002). The incidence rate of DMBA-induced mammary cancer in this experiment may also be explained partially in relation to other functional components in the date palm fruit such as phytoestrogens, phytomelatonin (Al-Qarawi et al., 2003), functional vitamins, minerals (such as Se and Mn), and non-nutritive functional compounds such as polyphenols, phenolic acids, carotenoids (Baliga et al., 2011), and antioxidants (Johnson, 2000; Vayalil, 2012) for example carotenoids, vitamins C and E, Se, Zn, flavonols such as catechins, ellagic acid, luteolin, quercetin, apigenin, *p*-coumaric, ferulic and sinapic acids, cinnamic acid derivatives, phenolic compounds,

polyphenols, and phytoestrogens (Kuhnle et al., 2009; Saafi et al., 2011) such as lignans, genistein (Vayalil, 2012).

The process of carcinogenesis involves inflammation (Poirier, 1987). It is thought that the anti-inflammatory effect of some food constituents might be protective against cancer such as carotenoids, the vitamins C, E and folic acid, Se, anthocyanins, ellagic acid, flavonoids (such as catechins), phenolic compounds, polyphenols, quercetin, genistein, phytic acid and resveratrol (Han and Liehr, 1992).

The date palm fruit might also protect against DMBA-induced mammary cancer by stimulation of immunity (Saarinen et al., 2002; Vayalil, 2012). Carotenoids, flavonoids such as quercetin, kampherol, isohamnetin (Fabian et al., 2010), daidzein (Heffelfinger et al., 2003), phytomelatonin (Tsuda et al., 2004) and conjugated linoleic acid (Chen et al., 2007) are known to be immunostimulants.

In DMBA-induced mammary cancer, TAM interferes with the tumour initiation (Russo and Russo, 1995). The results of tumour incidence data for AIN-93G+TAM are consistent with previous literature in terms of the fact that no study has demonstrated that TAM completely eliminates the appearance of mammary tumour (Fendle and Zimmiski, 1992). Unexpectedly, this group developed the highest incidence rate for DMBA-induced mammary cancer. This might be explained in relation to the resistance of TAM which occurred in many previous human and animal studies (Fendle Zimmiski, 1992; Han and Liehr, 1992; Chen et al., 2007).

Tumour latency

The promotion stage is defined as the time period starting after the carcinogen administration. This period is characterized by the growth of mammary tumours (Poirier, 1987). The later the palpable tumours appear, the lower the tumour growth rates are expressed (Aggrawal and Shishodia, 2006). The latency period of the DMBA-induced mammary cancer is a function of the promotion stage of carcinogenesis (Bobrowska-Korczak et al., 2012). In similar experiments to this study, the PIMDAC appeared between weeks 8-11 after DMBA administration. However, tumours continue to appear to 6-12 months after DMBA administration (Dillard and German, 2000). Differences between the results of the current study and other studies in terms of tumour latency might be due to the individual variation between rats and the experimental conditions to which animals were exposed such as the experimental diets and other external factors (Heffelfinger et al., 2003; Reiter et al., 2003). Unlike previous experiments, TAM delayed the appearance of DMBA-induced tumours. Results of the tumour latency could be explained partially with reference to the presence of the phytoestrogens in the date palm fruit. In similar researches, phytoestrogens were proved to regulate the expression of many related genes

such as cell nuclear antigen (*PCNA*), the antiapoptotic protein *Bcl-2* (Pugalendhi et al., 2010) and to enhance rat mammary gland differentiation (Teller et al., 1977; Chen et al., 2007).

The PIMDAC multiplicity, size and weight

In the progression stage of carcinogenesis, there is further metabolism of the carcinogen that leads to additional lesion(s), malignancy, appearance, and growth of tumours (Tsuda et al., 2004). The tumour growth is a function of numerous cellular mechanisms, including proliferation and apoptosis (Han and Liehr, 1992). Yet tumour growth is affected by tissue estrogen receptor expression (Molteni et al., 1995). The tendency for the date palm fruit to reduce tumour growth might be explained partially in relation to the functional components present in the fruit such as dietary fiber, Se (Jiang et al., 2009), and phytoestrogens (Molteni et al., 1995; Hall, 2001; Medina, 2001; Al-Qarawi et al., 2003; Jiang et al., 2009). Consistent with previous reports, it has been observed that TAM causes tumour shrinkage by reduction of cell proliferation (Fendle and Zimniski, 1992) and interferes with the phases of tumour promotion (Fendle and Zimniski, 1992).

Results of this research are in agreement with previous reports regarding the protective effect of soybean against mammary cancer (Constantinou et al., 2001). Although most of the "mammary cancer-related" functional components are present in soybean seeds in amounts greater than those present in the date palm fruit, the latter have exhibited protecting effect against this type of cancer.

Conclusion

It is concluded that the date palm fruit (*Phoenix dactylifera* L.) (at a level of 30% of the diet) inhibited the DMBA-induced mammary cancer at the initiation, progression, and promotion stages.

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Ethical standards

Conduct of this research at the Animal Unit of the Department of Nutrition and Food Technology of the University of Jordan was performed in compliance with the standards of the Deanship of Academic Research.

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