ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 10, Issue 7, 2017

Online - 2455-3891
Print - 0974-2441

Research Article

PREVENTIVE EFFECTS OF *PHOENIX DACTYLIFERA* POLYPHENOLS AGAINST 7,12-DIMETHYLBENZ(A)ANTHRACENE-INDUCED MAMMARY CANCER

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Received: 25 February 2017, Revised and Accepted: 01 April 2017

ABSTRACT

Objectives: Polyphenols are vital micronutrients, in our diet, which have a role in the prevention of progressive cancer and cardiovascular diseases developing. The main objective of this research was to evaluate the chemopreventive effects of some polyphenols of *Phoenix dactylifera* pits on 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary cancer of female albino rats.

Methods: The phenolics of *P. dactylifera* pits (Zahidi cultivar) were prepared by successive steps; extraction by ethanol:methanol:HCl:H_2O, adsorption chromatography using a silica gel column and preparative high performers chromatography. The cytotoxic activity of the phenolics was detected against human breast cancer cell line (MCF-7). The acetone phenolic fraction, 50 female albino rats, and DMBA carcinogen were used to study the preventive effects.

Results: The acetone phenolic fraction contained considerable amounts of the total phenolics and having antioxidant activity, which inhibit cancer cell line MCF-7 growth, and the inhibitory concentration 50% of cells (IC_{50}) at 24 h was 202 µg/ml for MCF-7 and 480 µg/ml for WRL-68. The purified phenolic compounds may be related to genistein and quercetin derivatives that inhibited MCF-7 cell line growth and IC_{50} were 1030 and 203.9 µg/ml, respectively, compared with negligible effects on normal cell line. The acetone phenolic fraction prevented mammary cancer formation in the DMBA-induced rat model.

Conclusions: The phenolics of date palm had chemopreventive effects against DMBA-induced mammary cancer, and they required further research to clarify the possible mechanisms that might have contributed to the preventive effects against mammary cancer.

Keywords: *Phoenix dactylifera*, Polyphenols, Chemopreventive, Mammary cancer.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10i7.18137

INTRODUCTION

Phoenix dactylifera L., ordinarily well-known as the date palm is a primal plant and has been cultivated for its edible fruit in the Arab world for centuries. *P. dactylifera* belongs to the Arecaceae family [1]; the fruits, pits (seeds) leaves, barks, pollens, and other parts of the tree are rich sources of carbohydrates, protein, amino acids, dietary fibers, phenolics, flavonoids, carotenoids, fatty acids, organic acids and certain vital vitamins and minerals. In addition to its dietary use, the dates are of medicinal applications and are used to treat a range of diseases in the numerous traditional systems of medicine [1-3]. Phytochemical investigations have shown that the fruits contain anthocyanins, phenolics, flavonoids, sterols, carotenoids, and procyanidins compounds known to have multiple valuable effects. Preclinical studies have revealed that the date fruits and pits possess free radical scavenging, antioxidants, antimutagenic, anticancer, anti-inflammatory, antimicrobial, antihyperlipidemic, nephroprotective, gastroprotective, hepatoprotective and immunostimulant activities, and sexual improvement [3-6].

Free radicals are generated by the metabolism of cells, especially in the mitochondria, these molecules are very effective and unstable, so they would cause many human health problems such as cancers and heart diseases. The use of antioxidants can scavenge free radicals directly or indirectly. Plants are a major source of natural antioxidants because they contain phenolic substances as secondary metabolites in plants which contribute to the growth and reproduction of plants [1,7].

Phenolic compounds are present in oil seeds and date fruits, and they are responsible for the dark colors and astringent flavors of some fruits. Date palm contains significant amounts of phenolic acids, flavonoids, carotenoids, and steroids [8,9]. There are three main families of phenolic compounds in dates, including flavonols, flavan-3-ols, flavan-3,4-diols; proanthocyanidins, and hydroxycinnamates. The hydroxycinnamates are the main group of phenolic compounds and can be found as derivatives of ferulic, synopsis, chlorogenic, and p-coumaric acids as well as their esters such as caffeoyl shikimic acid derivatives [9,10]. Flavonoids such as quercetin, luteolin, proanthocyanidins, and kaempferol are present in fresh dates. These are antioxidants and act as scavengers of free radicals, antimutagens, metal chelators, and signaling agents, and responsible for the protective effects on human health [11-13]. Quercetin has been confirmed to be an excellent antioxidant that can be used as an antiproliferative and anti-inflammatory. Quercetin has been used as a food supplementation, especially for people suffering from hypertension and sarcoidosis disease [14]. Kaempferol has a wide range of pharmacological activities, including antioxidant, anti-inflammatory, anticancer, antimicrobial, antiosteoporotic, antidiabetic, antiallergenic, neuroprotective, cardioprotective, and analgesic activities. It uses cytotoxic effects in numerous types of cancer and helps the body to fight cancer cells [15-17]. Luteolin has been revealed to inhibit tumor cell proliferation and angiogenesis [18].

Mammary cancer is a neoplastic modification in the epithelial cells of the mammary tissue. Most mammary cancers are hormone-dependent mammary cancer that developed depending mainly on the cell hormonal environment [19,20]. Estrogen-dependent mammary cancer is initiated when chemical carcinogen molecules such as 7,12-dimethylbenz(a) anthracene (DMBA) bind with the nuclear estrogen receptors [21,22] and then this complex interacts with the cell DNA at the estrogen

response elements. This reaction induces irreversible change in the cell's genetic which lead to the activation of proto-oncogenes and suppression of tumor suppressor genes that resulting an initiated cell. However, DNA alterations may occur due to the action of other physical or biological carcinogens [23].

In previous our results, the *P. dactylifera* phenolic extract was found the best source of antioxidant in comparing with other studied plant extracts because it had anticancer activity and more effective on breast cancer cell line MCF-7 than normal cell line WRL-68 (unpublished data). Hence, the objectives of this study were investigated the preventive effects of partially purified phenolics of the *P. dactylifera* pits against DMBA-induced mammary cancer.

MATERIALS AND METHODS

Raw plant material

In this study, the fruit pits of *P. dactylifera* of Zahdi cultivar was used as a source of phenolic compounds. The authentication of date palm (*P. dactylifera*) as follows:
Kingdom: Plantae
Phylum: Tracheophyta
Class: Liliopsida
Order: Arecales
Family: Arecaceae
Genus: Phoenix [1-4].

The fruit pits at tamor stage of ripening were collected, cleaned, and dried under the shade at room temperature and then ground and preserved in a clean container at 4°C for extraction.

Chemicals

All solvents, acids and some materials, including methanol (MeOH), ethanol (EtOH), acetone, hexane, ethyacetate, formic acid, glacial acetic acid, dimethyl sulfoxide (DMSO), HCl, silica gel, Cu (II), ammonium acetate and Na2CO3 were purchased from Gainland Chemical Co. Ltd., UK. The other materials were purchased from Sigma-Aldrich Co., USA, which including AlCl3, quercetin, gallic acid, Folin–Ciocalteu reagent, 3-[4, 5-dimethylthiazoyl]-2, 5-diphenyltetrazolium bromide (MTT dye), neocuprine, Trolox and potassium persulfate.

P. dactylifera phenolic extract preparation

P. dactylifera phenolic extract was prepared using the extraction system containing ethanol:methanol:HCl:H_2O at a ratio 50:29:1:20. The plant pits of Zahdi cultivar were crushed and milled to produce small particles (<2 mm). The plant sample (20 g) was extracted twice with the extraction system (400 ml) at the ratio of raw material to solvent 1:20 by soaking for 24 h at 30°C in shaker incubator (JSSI-200 Series, JSR, Korean). The plant extract was filtered under vacuum, concentrated in a rotary evaporator (IKA HB10, Germany) at 40°C, and then the concentrated extracts were stored at −24°C for further purification.

Phenolics of *P. dactylifera* purification

The resulting concentrated crude extract from *P. dactylifera* pits was partially purified using adsorption chromatography by silica gel (mesh 60-120) column. The slurry of silica gel was prepared by soaking with hexane [24-26], subsequently with absolute ethanol and then poured into the column (25 cm×2.5 cm) and washed with ethanol for 1 hr to obtain better packing. Concentrated plant extract (5 ml) was loaded into silica gel column and eluted successively with different solvents using batch ways (500 ml for each) 95% ethanol, 70% acetone, 30% acetone, and deionized water. The solvent was run at 4 ml/min and 5 ml fraction volume was collected. Frequently, each fraction was checked by absorbance at 275 nm for phenolic acids and isoflavonoids and 340 nm for flavonoids and coumestans using a spectrophotometer (PD-303 ultraviolet [UV], APEL Co., Ltd., Japan) [24-26]. then estimating phenolic and flavonoid compounds content. Finally, the positive fractions for each solvent were combined together and stored at −24°C for further analysis.

The dried sample of 70% acetone fraction was further purified by preparative high-performance liquid chromatography (PHPLC). They were dissolved in methanol, filtered through a 0.45 µm nylon filter, and injected (2 ml) into the PHPLC system (JASCO-FC2088-30)/Japan, on a C_{18} (300 mm×50 mm, 10 µm) column using mobile phase acetonitrile at a flow rate of 1 ml/min and with a detection wavelength of 254 nm [24-26].

Determination of total flavonoids

The total flavonoids were determined by the method described by Chaves *et al*. [27]. The extracts were diluted with distilled water to the 2 ml of each test, solution was added the same volume of 2% (w/v) $AlCl_3$ solution in methanol. This mixture remained undisturbed for 10 min before the ultraviolet (UV) spectrophotometric reading at 415 nm wavelength. The blank was prepared by replacing $AlCl_3$ solution by methanol. The total flavonoids were determined by the calibration curve using quercetin (Sigma-Aldrich) as standard and expressed in the µg equivalent of quercetin. The results were expressed as micrograms of quercetin/ml of the extract.

Total phenolic content

The Folin-Ciocalteu method was used to determine the total polyphenols, using gallic acid as a standard [27], a 1 ml of diluted test sample was added to 1 ml of 1 mol/l Folin–Ciocalteu reagent. This mixture remained undisturbed for 2 min before the addition of 2 ml of 20% (w/v) Na_2CO_3 solution and left undisturbed for 10 min. After that, the reading was performed by spectrophotometer (optizen pop - Korea) at 757 nm. The total phenolic content was determined by the calibration curve using quercetin gallic acid (Sigma-Aldrich) as standard and expressed in microgram equivalents gallic acid/ml of the extract.

Determination of total antioxidant capacity

Cupric reducing antioxidant capacity (CUPRAC) assay was performed according to the method of Apak *et al.* [28]. A 0.1 ml each of 10 mmol/l Cu (II), 7.5 mmol/l neocuprine, 1 mol/l ammonium acetate buffer (pH 7) solutions, and 0.06 ml water were mixed in a test tube. A 0.05 ml of diluted tested samples or Trolox standard solutions were added to the initial mixture. The absorbance was measured at 450 nm after 3-4 min. Trolox standard solutions were prepared at a concentration range from 40 to 400 µmol/l.

The total antioxidant capacity was calculated using the following equations:

$$An = At - Ar \quad (1)$$

$$x = \frac{y - b}{m} \quad (2)$$

$$\text{CRE's value} = x * 2189\ \mu\text{mol/l} \quad (3)$$

Where An=Net absorbance read; At=Absorbance of test sample after 3-4 min; Ar=Absorbance of the reference (0 min) at 450 nm. The second equation was the standard curve equation of the net absorbance versus Trolox concentration, where: y=y-axis value (optical density readings), m=Slope, x value coefficient=x-axis value (Trolox concentration µmol/l), and b=intercept. CRE's value=Copper reducing equivalents.

The results were expressed as total antioxidant CUPRAC mmol/l.

Cytotoxic activity by MTT assay

This assay was held at the Centre for Natural Product Research and Drug Discovery, Department of Pharmacology, Faculty of Medicine, University of Malaya/Kuala Lumpur, Malaysia.

To determine the cell viability by colorimetric assay using 3-[4,5-dimethylthiazoyl]-2,5-diphenyltetrazolium bromide (MTT dye), two kinds of cells were employed in this work: The human breast cancer cell line (MCF-7 cell line) and the normal human hepatic cells (non-tumorigenic fetal hepatic cell line WRL-68). Briefly, 100 µl

cell suspension was added onto the flat-bottomed micro-culture plate wells, each line in a separated plate, for the two cell lines and treated them with 100 µl partially purified or purified plant extract, incubated for 24 h, centrifuged to remove the dead cells. Aliquot of 100 µl from 2 mg/ml MTT dye was added to each well and incubation was continued for a further 4 h, then 50 µl of solubilization solution of DMSO was added into each well. The experiment was performed in triplicate. After complete solubilization of the dye, the absorbance of the colored solution obtained from living cells was read at 620 nm with an ELISA reader. The mean absorbance for each group of replicates was calculated. The percentage viability of cells exposed to various treatments was obtained as follows [29,30]:

$$\%\,\text{cell viability} = \left(\frac{\text{Mean absorbenace of treated sample}}{\text{Mean absorbenace of control sample}}\right) \times 100$$

The control was the nontreated cultures in all experiments that contained cells in the medium only.

Thin layer chromatography (TLC)

TLC was performed on silica gel G 60-grade absorbent plate, 20 cm×20 cm, [24-26]. Three solvent systems were used separately. The first one S_1=Ethylacetate: Acetic acid: Formic acid: H_2O (100:11:11:26), S_2=Hexane: Ethyl acetate: Glacial acetic acid (65:35:0.5), S_3=Ethylacetate: Ethanol: Acetic acid: H_2O (30:70:0.5:25), and S_4=Methanol: Acetic acid: H_2O at a ratio 40:1:50 (v:v:v). After running, the plates were dried at room temperature. The plates were kept in a TLC chamber containing 5% of $AlCl_3$ or iodine pellets or ammonium solution (2 ml) and were visualized under visible and UV light for fluorescence. Retention factor (R_f) was determined using the following equation:

$$R_f \text{ value} = \left(\frac{\text{Distance from baseline travelled by solute}}{\text{Distance from baseline travelled by solvent (solvent front)}}\right)$$

Spectroscopic characteristics

UV light spectra measurement

Small amount of the purified phenolic compound was dissolved in methanol, and the spectroscopy characteristic was determined at a range 200-800 nm using Shimadzu UV-visible (UV-VIS) spectrophotometer 1601 (Chemistry Department, University of Babylon).

Infrared (IR) spectra measurement

This analysis was performed at Chemistry Department, University of Al-Mostansiria. The IR spectrum of the purified phenolic compounds was measured by Perkin Elmer 2000 Fourier transform infrared (FTIR) uses a KBr discs technique in a range 600-4000/cm [24].

Animal experiments

Preparation of plant sample

The partially purified plant extract (500 mg) was dissolved 1 ml ethanol and then completed to 50 ml with distilled water at room temperature and was stored in the refrigerator until use.

Preparation of carcinogen

A carcinogen DMBA (Sigma-Aldrich, Germany) was freshly prepared at concentration 100 mg/kg body weight (BW) with a single dose of 20 mg of DMBA diluted in soy oil (2 ml) given the intragastrically by gavage method as described by Alfredo *et al*. [31].

Tumor induction

Animals

Female Wister albino rats (200±25 g) of 13±1 w age were selected for this study. They were obtained from the Animal House at University of Babylon, Iraq. The animals were bred under ideal conditions of temperature (26-29°C), humidity, and light, and they were fed with free access of water and food (pellets). All animals were housed in a controlled environment in steel mesh cages of six rats each on wood-chip bedding, for one week as an acclimatization period. Anesthetic procedures and handling of animals complied with the ethical guidelines of the Medical Ethical Committee of National Research Centre in Iraq, providing that the animals did not suffer at any stage of the experiment.

Experimental design

A total of 40 female albino rats were selected for this study and divided into five groups (10 rats each) (Table 1). Group 1: Normal, healthy control rats and Group 2: Considered as carcinogen reference which only received the carcinogen as a single dose of 20 mg of DMBA diluted in soy oil (2 ml) given the intragastrically by the gavage method as described by Alfredo *et al*. [31] to induced tumor and this group was left without any treatment. Group 3 was considered as the test group, each animal received a single dose of 20 mg of DMBA/rat given the intragastrically by the gavage method, after 15 d the animal orally administered 1.25 mg/kg BW (Body Weight) of partially purified phenolic extract of *P. dactylifera* pits (PDE) every 3 d for 104 d. Group 4 was considered as a drug reference for partially purified plant extract. It was normal rats and orally treated with 1.25 mg/kg BW of partially purified extract of *P. dactylifera* pits every 3 d for 120 d, according to LD_{50} which reveal the partial purified extract is safe till 2.5 mg/kg BW. All the animals of groups had free access to food and water.

We performed physical examinations, weekly. Each rat had six pairs of mammary glands that were checked by inspection, touching, and palpation.

For evaluation of the induction pattern, we performed two specific analyses at 8 and 13 w after drug ingestion. At the age of 211±7 d (15-17 w after DMBA), the animals were anesthetized with diethyl ether, and then the blood was collected by puncture of the heart and transferred to two clean and dry test tubes, one of them containing anticoagulant to prevent blood clotting and was used to examine blood parameters, the second tube left 10 min to clot and centrifuged at 3000 revolutions per minute (rpm) for serum separation. The separated serum was used for biochemical analysis of antioxidant analysis.

After the animals sacrificed, complete autopsies were performed, tumor, livers, kidneys, spleens, stomach, and intestines were collected. The mammary tumors were measured and weighed, and the findings recorded. The organs and tumors were divided into two portions. Representative fragments of the tumors and other organs (the first portion) were fixed at 10% buffered formalin in labeled containers. Tissues were processed routinely and embedded in paraffin wax. Sections of 5 µ thickness were cut, stained with hematoxylin-eosin (H&E) and examined under the light microscope [32].

The second portion of the tumor and organs was stored at −24°C for further analysis. As well as a portion of livers was weighed and homogenized in 5-10 volumes of bidistilled water by a ratio 1:10 w/v using electrical homogenizer, centrifuged at 4000 rpm for 15 min, the supernatants were collected, and placed in Eppendorf tubes, and stored at −20°C and used for determination of total antioxidant status of liver and serum. The homogenization was carried out as described by Newsholme [33]. The total antioxidant status was assayed in the liver tissue homogenate and serum [8,34].

Statistical analyses

Data were analyzed using SPSS version 22 software Fisher's exact with a significant $p<0.05$.

RESULTS AND DISCUSSION

The total phenolic compounds were extracted from *P. dactylifera* pits of Zahdi cultivar using organic solvent system, and results revealed that

Table 1: Animal experimental design of induction tumor and treatment in rat model

Groups (10 rats/group; BW=200±25 g)	Interval periods (d)	Treatment	
		Partially purified phenolics extract*0.25 mg/rat	Carcinogen (DMBA) 20 mg/rat
G : Control	1^{st}-120^{th}	-	-
G2: Carcinogen reference (DMBA)	1^{st}	-	Single dose
	2^{nd}-120^{th}	-	-
G3: Test (DMBA+PDE)	1^{st}	-	Single dose
	16^{th}-120^{th}	One dose/3 d	-
G4: Drug reference (PDE)	1^{st}-120^{th}	One dose/3 d	-

*LD_{50} of partial purified of plant extract=0.5 mg/rat. DMBA: 7,12-dimethylbenz(a)anthracene, PDE: Partially purified phenolic extract of *Phoenix dactylifera* pits

one gram dry weight (DW) of *P. dactylifera* pits powder gave 12-13% dry extract which contains 49.75±0.05 mg gallic acid equivalent (GAE)/g DW and 13.71±0.01 mg QE/g DW of the total phenolic compounds and flavonoids, respectively. The total antioxidant capacity of the extract depending on the CUPRAC assay was 6.60±0.02 mmol/l.

The fruits and pits of the date palm have been considered as a rich source of phenolic contents and correlated significantly with antioxidant activity. The total phenolic contents and antioxidant activities were variable depending on cultivars of dates because they can be affected by numerous factors such as growing conditions, geographic origin, cultivar, maturity of the tested dates and its pits, soil type, season, fertilizers, sampling, extraction method and conditions of storage [13,35-37,38], in addition to the type, structure and conformation of phenolic compounds [39]. Hence, our results of Zahidi cultirvar were in agreement or different with other previous studies, which revealed that the phenolic compounds of three native sun-dried date varieties from Oman (Mabseeli, Shahal, and Um-Sellah) and found; they have total phenolic compounds ranging from 3102 to 4430 mg GAE per 100 g fresh weight [40]. Another study was reported the phenolic acid composition in the pits of Mabseeli variety which contains p-hydroxybenzoic, protocatechuic, m-coumaric, ferulic, p-coumaric, vanillic, o-coumaric, gallic, and caffeic as the main phenolic acids with concentrations of 9.89, 8.84, 8.42, 6.93, 6.07, 4.07, 3.96, 0.28, and 0.18 mg of galic acid per 100 g fresh weight, respectively [40]. Furthermore, the phenolic contents of two date varieties, Deglet Nour and Medjol, were 661 and 572 mg of gallic acid per 100 g fresh weight, respectively [41]. Whereas, the phenolic contents of date were diverse depending on the sample types (part of the plants) such as fruits, seeds, fresh or dried date fruits, such as the comparative studies of Al-Farsi *et al*. [42] which performed on fresh and three dried date cultivars grown in Oman (Fard, Khasab and Khalas), and they revealed that the phenolic contents ranged from 134 to 280 mg ferulic acid equivalents per 100 g fresh weight. In comparison with the other studies that measured the phenolic contents of fresh dates of four cultivars (Allig, Deglet Nour, Kentichi, and Khouet Kenta) and found phenolic contents ranged from 209.4 to 447.7 mg GAEs per 100 g fresh weight [43].

The results of partial purification of *P. dactylifera* pits extract by adsorption chromatography using a silica gel column revealed that all used solvent fractions, including 95% ethanol, 70% acetone. 30% acetone and distilled water could be eluted the phenolic compounds at a percentage 4.81%, 12.32%, 2.23%, and 58%, respectively, whereas the total flavonoids were soluble only in organic solvents but not in distilled water, and they were eluted at a percentage 1.06%, 67.08% and 8.63% in 95% ethanol, 70% acetone and 30% acetone, respectively, in other word, the most of the phenolic contents of *P. dactylifera* was eluted by distilled water (soluble in water) and the most flavonoid content was eluted by 70% acetone. The partially purified of *P. dactylifera* extract contained 150 mg GAE/g DW of the total phenolics; the total flavonoids was 10.5 mg QE/g DW and the total antioxidant capacity 5.84±0.03 mmol/l. The 70% acetone fraction of phenolics was selected to further purification by preparative HPLC-UV (K-2501 UV detector). The best separation conditions were gained using the mobile phase acetonitrile at a flow rate of 1.0 ml/min and with a detection wavelength of 254 nm. 36 peaks appeared at a retention time ranged from 0.262 to 54.694 min with total area 38944673 (Fig. 1), out of these peaks only two peaks (8 and 9) which appeared at a retention time 5.637 and 6.702 min had area 4718475 and 34165367 with height 120410 and 699606, respectively. That indicates the most phenolics of the partially purified acetone fraction composed of two compounds and others were traces. The two peaks (8 and 9) were selected to assay the cytotoxic activity against two cell lines (MCF-7 and WRL-68).

Our results were in agreement with previous studies that purified the phenolic compounds by adsorption chromatography using a silica gel column and HPLC techniques [25,26,44]. There are many techniques for phenolics purification depending on the type of these compounds and their chemical structures. In our study, we focus on phenolic acid and flavonoid compounds of *P. dactylifera* that were hydrophobic phenolics as antioxidants because we search for anticancer substances depending on the references that reported the flavonoid compounds of *P. dactylifera* had anti-inflammatory and anticarcinogenic effects and it decreases the fragility of blood vessels such as rutin (also known as vitamin P) [45-48], quercetin, and other phenolic acids [9] which had a preventive effect against breast cancer [31].

The cytotoxic activity of the 70% acetone phenolic fraction of *P. dactylifera* pits (designated PDE) and the purified fractions of peaks 8 and 9 (designated as A and B compounds) were assayed using two cell lines, including breast cancer cell line (MCF-7) and the normal human hepatic cells (non-tumorigenic fetal hepatic cell line WRL-68). The results appeared that PDE had a cytotoxic effect on the cancer cell line MCF-7 and the inhibitory concentration 50% of cells (IC_{50}) at 24 h was 202 µg/ml (156.91 µg phenolics) for MCF-7 and 480 µg/ml (372.86 µg phenolics) for WRL-68. Whereas, the purified phenolic compound (B) which represents peak 9 (retention time 6.702 min) had cytotoxic activity against both cell lines and the IC_{50} of cell lines MCF-7 and WRL-68 equal 203.9 µg/ml and 1141.4 µg/ml respectively (Table 2). While the phenolic compound (A), peak 8 (retention time 5.637 min), had low activity against the MCF-7 cell line (IC_{50}=1030 µg/ml) and negligible activity against WRL-68 cell line, that indicated the purified phenolic compound (B) was more active than partially purified phenolics (70% acetone fraction) which containing two compounds and other impurities which had may be a negative effect on cytotoxic activity on both cell lines. These results were in agreements with Abou-Elella and Mourad [48] showed that *P. dactylifera* seed extract had anticancer activity against Ehrlich ascites carcinoma cells. Furthermore, the date fruit extract had preventive effects against DMBA-induced mammary cancer in rats, and it is related to the effect on the hormone 17-β-estradiol [31]. It can be used against several kinds of tumor and has been found to exhibit a dose dependent anticancer activity [31,48].

As a result the potential purified phenolic (A) and (B) compounds were selected to characterize by TLC as shown in Table 2. The results of the purified phenolic compounds revealed that the R_f of compound A was 0.82 (bright yellow spot under UV) and the compound B had R_f=0.84 (bright yellow spot under UV) using mobile phase methanol:acetic acid:water at a ratio 40:1:50 (v:v:v) in comparison with the quercetin which had R_f=0.80. These results consistent with previous studies [49,50].

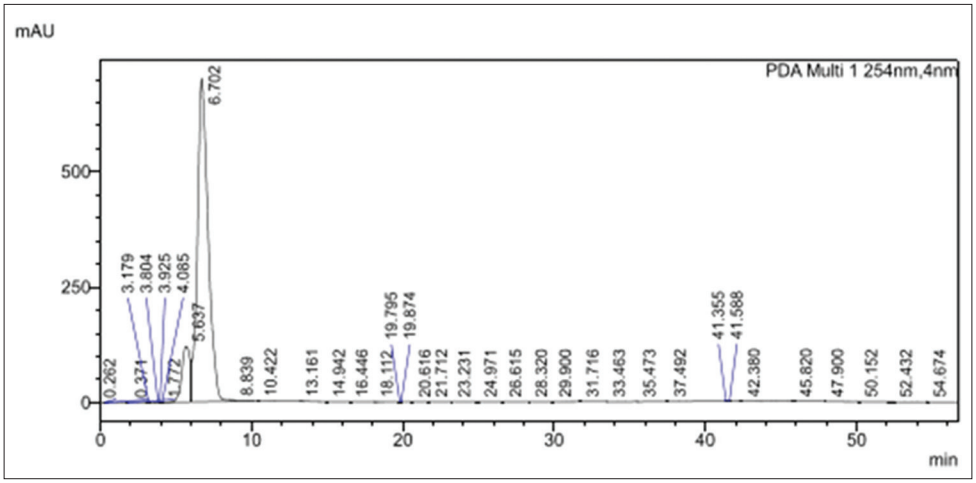

Fig. 1: Preparative high performance liquid chromatography chromatogram of the partially purified of *Phoenix dactylifera* seeds extract

Table 2: The quantitative and qualitative analysis of purified phenolic fractions of acetone extract of *P. dactylifera* pits

HPLC peaks	R_t (min)	Cytotoxic activity IC_{50} (µg/ml)		Total phenolics (µg GAE/ml)	Total antioxidant CUPRAC mmol/l	TLC (R_f)	λ_{max} (nm)	Related phenolic derivatives
		MCF-7	WRL-68					
PDE	-	202	480	156.91±0.13	5.84±0.03	0.82 and 0.84	254,300	Mixture
P8 (A)	5.637	1030	Very wide	108±1.24	3.98±0.15	0.82	325	Genistein
P9 (B)	6.702	203.9	1141.4	102±0.57	4.21±0.22	0.84	305,370	Quercetin

PDE: 70% acetone fraction of partially purified extract of *P. dactylifera* pits, R_t: Retention time, HPLC: High performance liquid chromatography, CUPRAC: Cupric reducing antioxidant capacity, TLC: Thin layer chromatography, *P. dactylifera: Phoenix dactylifera*, R_f: Retention factor, GAE: Gallic acid equivalent

The results of the UV/VIS λ_{max} of the compounds, which were dissolved in 95% ethanol+$AlCl_3$+potassium acetate, revealed that the compound (A) had a λ_{max} in 235 nm, 273 nm, 311 nm and 371 nm, whereas the compound (B) two λ_{max} in 235 nm, 274 nm, 305 nm, 374 nm and 414 nm. These results consistent with the observance of genistien and quercetin derivatives, which had λ_{max} in 272, 306, 371 nm for genistein 8-C-β-D-glucopyranoside and λ_{max} in 274, 303, 421 nm for quercetin 3-O-β-D-glucopyranoside were isolated from date seeds [43,51,52] that may indicate the compounds A and B were genistien and quercetin and their derivatives, where the phenolic acids such as genistin that detected in 213, 239 (shoulder), 332-370 and λ_{max} 325 nm. Also, chlorogenic (243, 325 nm), caffeic; 220, 240 (broad), 294 (pre-shoulder) and λ_{max} 325 nm. Whereas, ferulic was detected in 218, 236 (broad), 294 (pre-shoulder), 324 nm and λ_{max} 325 nm, and sinapic (238, 326 nm and λ_{max} 325 nm) [9,40,53].

Results of FTIR - spectrum of phenolics revealed that the absorption bands of functional and/or structural groups of this spectrum as shown in Fig. 2 and Table 3. The active purified compounds A and B of date pits phenolics presented the bands showed in Fig. 2. It can be observed broad bands for both compounds in a range 3600-3200/cm belongs to stretching vibration of the phenolic hydroxyl group (-OH) which represent to hydrogen bonding [54]. Bands approximately in the same range of wavelength in different phenolic compound were also identified, e.g., a band at 3312.65/cm, 3323.61/cm and 3285/cm, related to stretching (ν) vibration of hydroxyl groups [55,56] and a band at 3433/cm, related to OH wagging (OH of phenolic compounds) [57]. The band at 2154.49/cm may be related to C≡C-C of alkynes [56] or may be related to SH stretch, CO stretch or SiH a-stretch [58-60]. Whereas the appearance of two mediums and weak bands at 1625.99/cm and 1500/cm stretching vibration of aromatic (C=C) group. The band at 1625.99/cm could be due to stretching vibration of C=C groups [55], due to aromatic ring deformations [56], due to flavonoids and amino acids: Stretching vibration of C=O and of C=C, asymmetric bending vibration of N–H [54], due to C=O stretching vibration of caffeic acid and its derivatives [61,62] and/or due to stretching vibration of C=O of lipids and flavonoids [55,56]. The bands at 1238.3/cm and 1049.28/cm would be due to vibration of the C–O group of hydroxyl flavonoids [56] or related to C-OH stretch, C-O stretching, CH deform [57-65].

These compounds had different retention time in HPLC chromatogram and FTIR and UV spectra that indicate these two different compounds may be related to quercetin derivative of the compound B and the compound A related to genistein derivatives due to it was consistent with Pandit and Patravale [65] who studied genistien. Hence, further research will be needed to find out the structural analysis of phenolic compounds (A and B) by the use of different analytical methods such as nuclear magnetic resonance and mass spectrophotometer.

Animal experiments

Breast tissue may be the main goal of the toxic effects of a variety of lipophilic carcinogens such as polycyclic aromatic hydrocarbon (PAH) [65]. DMBA is a member of the PAH family, the metabolic activation of this compound produces radical cations, free radicals, and oxygenated metabolites [66]. Sequentially, the oxidative stress produces harmful effects by initiating lipid peroxidation [67]. In general, therefore, DMBA can induce extensive oxidative damage in various bodily organs (especially liver and breast), a property that has made DMBA an appropriate and useful agent to generate *in vivo* models of rat breast cancer [68,69]. The carcinogenicity of DMBA in rats was accompanied by substantial increases in the activities of antioxidant enzymes, as a response to the induced oxidative stress and production of reactive oxygen species [70]. DMBA produces DNA-reactive species during their metabolism, which may promote oxidative stress in cells. In the cell, the reactive metabolite DMBA-3, 4-dihydrodiol-1,2-epoxide (DMBA-DE) adds adenine and guanine residues in DNA. The conversion of genotoxic metabolites as DMBA-DE is promoted by the action of the cytochrome P450 family. CYP1A1 and CYP1B1 are identified as the enzymes that metabolize DMBA to produce DMBA-DE [71]. Subsequently, it has disrupted vital cellular functions by damaging proteins and lipid membranes. As a result, these changes induced developing leukemia and anemia [72,73].

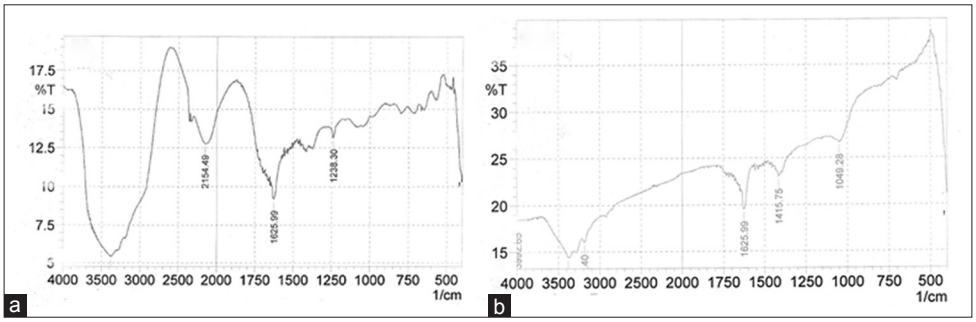

Fig. 2: (a-b) Fourier transform infrared spectra of the purified active fractions of *Phoenix dactylifera* pits extract

The compound (A) contained the peaks: C-OH at 1238.30/cm, aromatic C=C and C=O at 1625.99/cm, Alkyne C≡C-C at 2154.49/cm, phenolic O-H group at 3379.29/cm. The compound (B) contained the peaks: C-OH stretch in 1049.28/cm, C–O/C–H bending at 1415.75/cm, aromatic C=C and C=O at 1625.99/cm, phenolic O-H group at 3381.21/cm and O-H stretch at 3186.40/cm.

Table 3: The functional groups of purified active substances of *P. dactylifera* pits extract determined by FTIR

Peaks	Wavenumber (cm^{-1})	Bond	Functional group	Group band (cm^{-1})	Reference
1 (B)	3381.21	H- bounded of phenolic O-H		3600-3200	[54-57]
2 (A)	3379.29	Alcohol/Phenol			
3 (B)	3186.40	O-H stretch			
4 (A)	2154.49	C≡C-C	Alkyne	2260-2110	[55-60]
		SH stretches		2154	
		CO stretch		2153.8	
		SiH a-stretch		2154	
5 (A&B)	1625.99	C=C stretch	Aromatic	1675-1600	[64,60]
		C=O stretching mode	Ketones	1675-1625	
6 (B)	1415.75	C–O/C–H bending		1421–1415	[60]
7 (A)	1238.30	C-OH stretch	Ether	1300-1000	[49,55]
		C-O stretching	Ether	1275-1200	[57-63]
		CH deform		1237.70	
8 (B)	1049.28	C-OH stretch	Ether	1300-1000	[54,55]

FTIR: Fourier transform infrared spectra, *P. dactylifera: Phoenix dactylifera*

In our results, the female rat groups were received the chemical carcinogen DMBA at the age of 91-98 d. After 12-21 w, the animals revealed variable phenotypic characteristics including 50% of them formed tumors (DMBA-induced mammary tumor), as well as all DMBA-rat tongue converted to the dark-gray to black color and their eyes suffering from bleeding (Fig. 3). The rate of tumor transformation was very low in comparison with the previous studies that documented that the rat groups were received the carcinogenic agent at the age 57 d. Because of the susceptibility of the mammary gland to DMBA carcinogenesis is strongly age-dependent, being maximal when the drug is administered to rats between the ages of 45 and 60 d, which is the age of the beginning of sexual maturity [74].

In the present study, the DMBA-PDE and PDE-reference groups were orally treated with 1.25 mg/kg BW of partially purified extract of *P. dactylifera* pits every 3 d for 120 d; the LD_{50} PDE was 2.5 mg/kg BW. In other word, each animal received 0.25 mg of the PDE which containing 37.5µg of total phenolics and 2.6 µg of total flavonoids and the LD_{50} PDE was 75 µg of total phenolics and the total antioxidant capacity was 3.41±0.01 mmol/l.

The survival, ages of rats were different among groups like DMBA-reference group was survival for 7-12 w, whereas DMBA-PDE group (test group) was survival for 16-21 w in comparison with the control and PDE-reference groups were survival until end the experiment (21 w). That may be indicating the partially purified PDE phenolics enhanced rat health and immunity and the rats became more active in comparison with the DMBA - reference group as a result significantly prolongs the lifespan of rats, also the symptoms of bleeding eyes and dark-tongue gradually disappeared after rats treated with PDE phenolics. In addition to the DMBA treated group showed a highly significant decrease ($p<0.05$) in BW gain compared to the control group) as shown in Fig. 4. The obtained results were in agreement with the findings of Mathivadhani *et al*. [75]. The loss in BW of rat was in agreement with Devlin [76] who reported that the weight loss in the treated rats is largely from skeletal muscles and adipose tissue with relative sparing of visceral proteins. Otherwise, DMBA+PDE treated group displayed a highly significant increase in BW when compared to DMBA treated group and this may be due to the antioxidant activity of *P. dactylifera*. However, *P. dactylifera* is ranked one of the plants with highest antioxidant values [77].

After sacrificing animals found that many changes in blood parameters and histopathology characteristics of some organs such as the spleen, liver, kidney, and digestive system in comparison with control. In spite of the DMBA exposed rats (DMBA reference group) became very slim, they collected fats in the abdominal cavity and formed tumors (50% rats) in different sizes ranged from 1.2 to 3 cm with weight ranged 1-3.1 g. The DMBA carcinogen induced different types of tumor, including fat cysts, connective mass tissue and mammary gland carcinoma (Fig. 5). Whereas the other groups had not any tumor, but both DMBA reference group and the DMBA-PDE group suffered from anemia.

As an indication of stress, the use of hematological methods provides valuable knowledge about physiological reactions occurring against changing environmental conditions, particularly understanding the physiological and hematological changes occurring at sublethal toxicity, to expect the possible level of threat to life. In this study, a

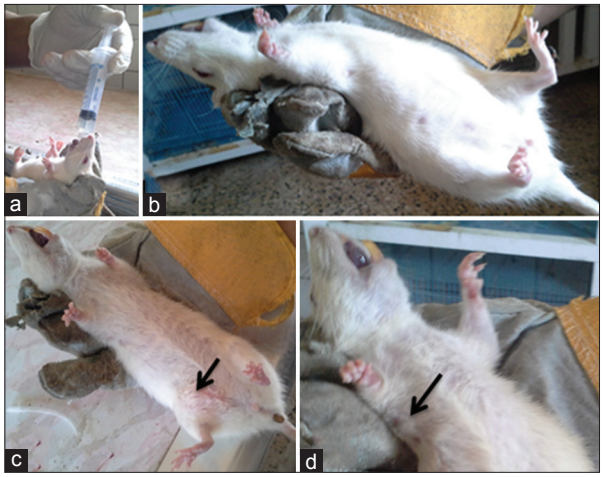

Fig. 3: Animal experiments using Female Wister albino rats (200±25 g) of 13±1 w age treated with DMBA carcinogen and partially purified phenolics agent given the intragastrically by the gavage method (a); control (b); tumor formation with change tongue color to dark-gray to black (c and d)

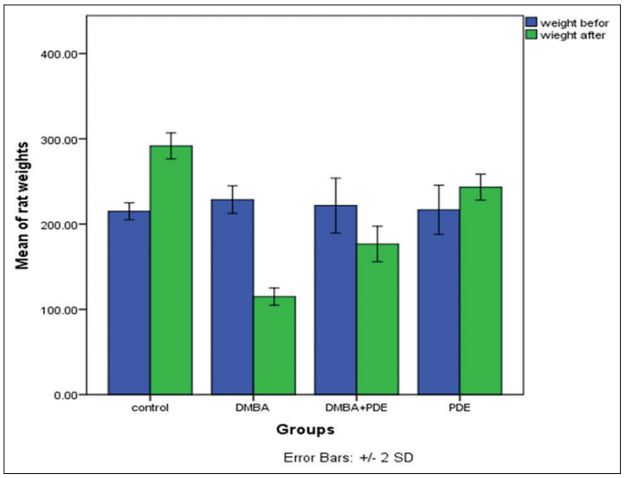

Fig. 4: Rat body weights before and after treatment with carcinogenic agent DMBA and drug partially purified phenolics of date pits

Values are expressed as mean±standard deviation (SD) (n=6). Statistical significance was done by oneway analysis of variance (ANOVA) followed by least significant difference (LSD). The mean difference is significant at the 0.05 level (p<0.001) of rat weights after treatments. Dunnett t-tests (2-sided) treat one group as a control, and compare all other groups against it.

statically significant increase (p<0.05) in total leukocyte count (white blood cells [WBC]), eosinophil, neutrophil, and monocyte values for rats exposed to DMBA as [73] have been observed. However, there was a statistically significant increase in lymphocyte value (p<0.05), and not significant changing basophil value (p<0.05), as shown in Table 4.

In addition, high leukocyte values depending on DMBA toxicity result from the stimulation property of this toxic and depressive agent on the immune system [78,79]. The toxic effect of DMBA caused a stress on the rats. With administration of partially purified PDE-phenolics, the toxic effect of DMBA decreased statistically significantly (p<0.05) (Table 4). Statistically significant decreases in hemoglobin and hematocrit values for rat exposed to DMBA have been shown [73]. However, there was a statistically significant decrease in mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values, and, in addition, not significantly changing magnesium hydroxy-carbonate hydrate (MHCH) and erythrocyte values (p<0.05 and Table 4). With administration of PGE-phenolics, the toxic effects of DMBA on MCV and MCH values could be prevented significantly (p<0.05 and Table 6). Higher red cell distribution values indicate greater variation in size of erythrocyte, anemia resulting from an inability of the bone marrow to produce red blood cells.

In essence, a decrease in hemoglobin and hematocrit values can be an indicator of anemia, subsequently resulting in inhibition of erythropoiesis in the hemopoietic organism [80], Furthermore, decrease in MCV value showed that anemia was a microcytic anemia monochrome type because there was no change in MHCH value.

It is known that oxidative stress can change hematological parameters such as erythrocyte number, hemoglobin amount hematocrit value, and total leukocytes.

The blood parameters of rat groups appeared that the groups received carcinogenic agent DMBA suffered from lymphoma, anemia and increasing in platelet numbers (Table 4) which indicated these animals suffering from cancer like leukemia. The rat group received the partially purified phenolics drug after carcinogen, they were becoming more active than DMBA reference group and their blood parameters were enhancing gradually through 4 months that indicated that the drug enhancing animal immunity, also they may be required long time of drug treatment for healing. DMBA carcinogen could not induce tumor in the DMBA-PDE group may be partially purified PDE was stopped the multiplying mammary cells due to prevention effects in comparison with control group DMBA reference because of the partially PDE of date pits are very rich in antioxidants and has the estrogen-like activity that bind to estrogen receptors in mammary cells and prevent DMBA from binding with these receptors [77]. Phenolics and flavonoids such as ginestin and quercetin have protective effects in estrogen-dependent breast cancer by binding to estrogen receptor and modulating estrogen metabolism by selective inhibition of CYP1B1 activities without affecting CYP1A1 or CYP1A2 [81]. Since flavonoids inhibit DNA binding, they have antimutagenic, anticlastogenic, antitumor, and anticarcinogenesis effects [82-85]. The above results were in agreement with previous studied that documented that the DMBA carcinogen induced leukemia in rats by its effects as a mini - nucleus and induces single- and double-strand breaks in nuclear and mitochondrial DNA [72]. In addition to forming DNA adducts, oxidative products resulting from mutagen metabolism, such as DMBA, has disrupted vital cellular functions by damaging proteins and lipid membranes. As a result, these changes induced by the chemical carcinogen DMBA, was reported to be developing leukemia and anemia [72,73].

When the measurement of the total antioxidant status of serum and liver concentrations were estimated of rat groups, the results appeared that the total antioxidant status in serum had no significant variation in comparison with liver that appeared a significant variation (p<0.05) between DMBA and DMBA+PDE groups compared with control group of liver antioxidants (Fig. 6) that indicate the amount of free radicals in liver rats of DMBA reference group were higher than DMBA+PDE group, as a result of inhibiting of antioxidant enzyme activities by DMBA carcinogen, and these results were in agreement with the previous studies [85,86].

The total antioxidant status assay is practically measures nonprotein total antioxidant capacity. However, proteins constitute the main antioxidant component of serum (plasma). The randox-total antioxidant status assay can determine the antioxidative effects of bilirubin, vitamin C, uric acid, ascorbic acid, glucose polyphenols, and proteins such as transferrin and albumin, whereas the total antioxidant status assay in the liver is measured protein total antioxidant capacity [87]. The liver is the central organ of metabolism and acts as an organ of storage. Hepatic cells metabolize many

potentially toxic substances. The greater susceptibility of liver to damage by chemical agents is presumably a consequence of its primary role in the metabolism of foreign substances [88]. The liver is thus a versatile organ involved in drug metabolism and detoxification, hence the antioxidant enzymes and other parameters were estimated in the liver as well as serum. The reduced form of glutathione (GSH) is a biological antioxidant present in high amounts, especially in the liver and its presence is prerequisite for protection against oxidative damage. Decreased levels of GSH accompanied by decreased activities of antioxidant enzymes superoxide dismutase (SOD), glutathione S-transferase (GST), catalase, and glutathione reductase (GR) show the poor antioxidant status of rats treated with either stress or DMBA alone or in combination. The decreased levels of GSH and GST as observed in restrained rats might help in DMBA-induced carcinogenesis by decreasing its clearance from the system [88]. The extracellular fluids of animals, such as blood plasma and tissue fluid, contain little or no catalase activity and only low activities of SOD. There is also very little reduced GSH in most extracellular fluids-about 20 mM in the plasma of rats. Therefore, the major antioxidants found in blood plasma are ascorbic acid, uric acid and glucose, in addition to transferrin and albumin. Ascorbic acid has multiple antioxidant properties, including an ability to regenerate a-tocopherol by reducing a-tocopheryl radicals on the surface of membranes. Uric acid can act as an antioxidant both by binding to the radicals and by directly scavenging oxidizing species. It, therefore, inhibits lipid peroxidation. Glucose also acts as a scavenger of hydroxyl radicals [89]. The levels of all these antioxidants were found to be decreased with an increase in lipid peroxidation in plasma of rats exposed either to stress alone or DMBA alone.

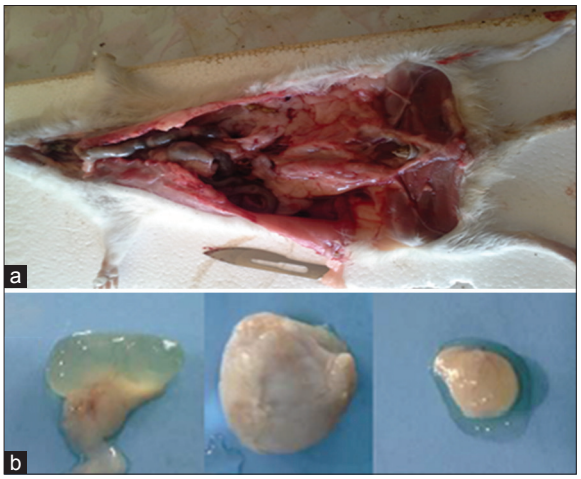

Fig. 5: The sacrificing and complete autopsies of animal appeared fat aggregate in abdomenal cavity (a), and (b) the tumor mass formation in DMBA-control group, including from the lift to right fat cysts, connective mass tissues and mammary gland carcinoma

CONCLUSION

The date palm *P. dactylifera* pits in addition to fruit had protective effects against DMBA-induced mammary cancer in the initiation, promotion, and progression stages of DMBA-induced mammary carcinogenesis. These effects are required further research to clarify the possible mechanisms that might have contributed to the preventive effect of this fruit against mammary cancer.

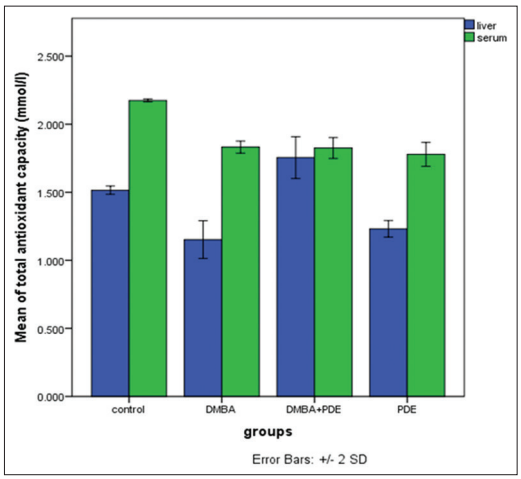

Fig. 6: The total antioxidant capacity of serum and livers of experimental rat groups treated with 7,12-dimethylbenz(a) anthracene (DMBA) carcinogen and the partially purified phenolics of date pits. Values are expressed as mean±standard deviation (n=6). Statistical significance was done by one way ANOVA followed by least significant difference. The mean difference is significant at the 0.05 level (p<0.000-0.001) for DMBA and DMBA+partially purified phenolic extract of *Phoenix dactylifera* pits groups compared with control group of liver total antioxidant capacity and they were not the significance of serum total antioxidant status. Dunnett t-tests (2-sided) treat one group as a control, and compare all other groups against it

Table 4: Effect of *P. dactylifera* extract on Hematological parameters in control and experimental rats

Blood Parameters	Group I Control	Group II DMBA control	Group III DMBA+PDE	Group IV PDE control	Lower-upper limits
WBC (10^3/µl)	9.70±2.79	6.12±2.39	7.64±4.01	8.50±3.08	4.00-12.00
LYM %	41.90±9.02	77.08±6.85*	58.51±9.47*	38.00±9.89	25.00-50.00
MON %	7.70±2.54	10.20±1.06	15.73±2.93*	6.83±2.63	2.00-10.00
GRA %	65.90±11.58	13.00±5.66*	25.89±7.13*	61.67±12.90	50.00-80.00
HCT %	45.70±7.25	33.70±0.62*	32.90±1.67*	44.50±9.24	35.00-55.00
RDW %	13.80±2.20	16.65±1.07*	17.16±0.99*	12.83±2.31	10.00-16.00
RBC (10^6 µl)	5.32±0.86	5.74±0.01	5.38±0.43	4.92±0.91	4.00-6.00
HGB (g/dl)	14.15±2.66	10.82±0.07*	10.68±0.56*	13.08±2.69	11.00-17.00
MCV (µM^3)	90.70±9.29	58.72±1.29*	61.88±8.93*	87.17±9.26	80.00-100.00
MCH (pg)	30.80±2.93	18.83±0.18*	20.11±2.82*	29.67±3.26	26.00-34.00
MCHC (g/dl)	33.10±1.77	32.03±0.33	32.49±0.25	32.92±1.85	31.00-35.50
PLT (10^3/µl)	315.00±96.75	787.33±59.90*	604.67±65.72*	283.33±112.72	150.00-400.00
MPV (µM^3)	9.40±1.59	5.99±0.13*	5.90±0.39*	8.92±1.74	7.00-11.00

Values are expressed as mean±SD (n=6). Statistical significance was performed by one-way ANOVA followed by LSD. *The mean difference is significant at the 0.05 level (p<0.01-0.001). Dunnett t-tests (2-sided) treat one group as a control, and compare all other groups against it. *P. dactylifera: Phoenix dactylifera*, LYM: Lymphocyte, PDE: *P. dactylifera* extract, DMBA: Carcinogenic gent 7.12-dimethylbenz[a] anthracene, WBC: White blood cells, RBC: Red blood cells, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, RDW: Red cell distribution width, PLT: Platelets, MPV: Mean platelet volume, MON: Monocyte, GRA: Granulocyte

ACKNOWLEDGMENT

The authors are thankful to Pioneer Projects Department, Research and Development Directorate, Ministry of Higher Education and Scientific Research, Iraq, for funding this research which was done at the Laboratory of Biotechnology and Genetic Engineering, Biology Department, College of Science, University of Babylon, Iraq.

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