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Modulatory Effects of Date Palm (*Phoenix dactylifera L.*) Pits Extract Against Ethinylestradiol Induced Genotoxic Damage in Cultured Human Lymphocytes

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Abstract

Ethinylestradiol is widely used in oral contraceptive formulations and also for the treatment of various sexual and metabolic disorders. It is not only a genotoxic agent but also a tumor initiating agent. In the present study, the modulatory effect of aqueous extract of date pits was evaluated against the genotoxic effect induced by ethinylestradiol on human lymphocytes using chromosomal aberrations (CA), blast index (BI), mitotic index (MI), sister chromatid exchanges (SCE) and replication index (RI) as parameters. The date pits extract was evaluated at $1.075x10^{-4}$, $2.125x10^{-4}$, $3.15x10^{-4}$ and $4.17x10^{-4}$ g/ml along with 10 µM of ethinylestradiol in culture medium. The results showed a significant dose-dependent decrease in the frequency of CA and SCE induced by ethinylestradiol. The results of the present study suggest that the plant extract *per se* does not have genotoxic potential, but can modulate the genotoxicity of ethinylestradiol in cultured human lymphocytes.

Keywords: Ethinylestradiol, Phoenix dactylifera, Genotoxicity, Chromosomal aberrations, Sister chromatid exchanges, Human lymphocytes.

الفعالية المُعدِّلة لمستخلص نوى التمر ضد عقار Ethinylestradiol الذي يسبب ضرراً بالمادة الوراثية للخلايا اللمفاوية البشرية المزروعة

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الخلاصة

يستخدم عقار Ethinylestradiol على نطاق واسع مانعاً للحمل يؤخذ عن طريق الفم، ويستعمل في علاج بعض الاختلالات الجنسية والأيضية، لكن هذا العقار ليس ساماً للمادة الوراثية في الخلية فحسب، بل يعتبر عاملاً محفزاً للاورام. في هذه الدراسة تم تقييم فعالية المستخلص المائي لنوى التمر ضد التأثير السمي على المادة الوراثية تجاه الخلايا اللمفاوية البشرية بفعل عقار Ethinylestradiol ، وذلك من خلال بعض المقاييس الوراثية التي شملت: دراسة التغيرات الكروموسومية، معامل التحول الأرومي، معامل الانقسام الخيطي، التبادل الكروماتيدي الشقيق و معامل التضاعف. أظهرت النتائج إن المعاملة بالتركيز 10 مايكرومولار من عقار Ethinylestradiol مع التراكيز $1,075\times10^{-4}$، $2,125\times10^{-4}$، $3,15\times10^{-4}$، $4,17\times10^{-4}$غرام/مللتر من مستخلص النوى في الوسط الزرعي أظهرت إنخفاضاً معنوياً معتمداً على التركيز في التاثير السمي المُستحث بفعل العقار. لقد بيّنت نتائج هذه الدراسة أن التراكيز المستخدمة من مستخلص النوى لم تبدي تأثيراً سمياً على الخلايا اللمفاوية، بل عدّلت التأثير السمي للعقار تجاه هذه الخلايا.

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Introduction

Date palm pits have not generally received much attention as antioxidant sources and this could be due to their lack of popularity and lack of commercial applications. However, there are considerably higher ratios of by-products arising from fruit-processing plants and derived products have experienced growing worldwide popularity [1].

Worldwide dates production has increased exponentially over the last four decades. Out of the total world production of 7.4 million tons of dates, 5.4 million are coming from the Arab World [2].
The date palm (*Phoenix dactylifera* L.) has been an important crop in Iraq and Middle Eastern countries. These countries are the Favourable areas in cultivation of this crop, where 60 million date palms located there. In Iraq, the date palm cultivated area is about 125,000 ha. [3]. For Muslims all over the world, dates are of religious importance and are mentioned in many places in the Holy Quran. They are customarily used to break the day long fast during the holy month of Ramadan [4]. Therefore, utilization of date by-products, particularly pits, is very important to date cultivation and to increase the national income to this sector which supports over one million people. Recently, date pit powders are marketed and are a source of choice to people preferring a non-caffeinated coffee with coffee-related flavor [5].

Experimental studies have shown that feeding rats with the aqueous extract of date flesh or pits exhibit gastroprotective, hepatoprotective and nephroprotective effects in rodents [6-8]. The date pits have also been reported to exhibit anti-aging properties and significantly reduce skin wrinkles [9]. Moreover, date pits were grind and added to the feed of domesticated animals to enhance growth and this was ascribed to an increase in the plasma level of estrogens or testosterone [5, 10,11].

Estrogens are used for treating many types of sexual disorders and as part of various oral contraceptive formulations [12]. There is sufficient evidence for estrogen carcinogenicity and genotoxicity in various experimental models [13]. Ethinylestradiol (EE) is commonly used in oral contraceptives and in other drug formulations [14]. The prolonged use of oral contraceptives has been reported to induce various types of cancers [13]. There are also reports of the genotoxicity of ethinylestradiol in various experimental models [15-19]. Accordingly, the present investigation is a novel attempt to spotlight on the efficacy of the aqueous date pits extract for reducing DNA damage induced by ethinylestradiol in cultured human peripheral lymphocytes using cytogenetic parameters including blast index (BI), mitotic index (MI), chromosomal aberrations (CA), replication index (RI) and sister chromatid exchanges(SCE).

Materials and Methods

Chemicals:

Ethinylestradiol (CAS: 57-63-6; Sigma-Aldrich); RPMI 1640, Fetal calf serum, Phytohaemagglutinin-M, Antibiotic-antimycotic mixture (Gibco, Invitrogen), 5-Bromo-2-deoxyuridine, Colchicine (Sigma-Aldrich); Dimethylsulphoxide (SRL, India); Giemsa stain (Merk).

Preparation of date pits extract (DPE):

Date fruits were purchased from a local market in Baghdad, Iraq. The pits were manually separated from the flesh rip fruit and washed clear of any flesh by water. The dried date pits were oven dried for seven days at 60°C and then finely ground into powder using a stainless-steel blender. The water extract of the date pit of 100 g was made by adding distilled water to coarsely pounded date pit (1:2 ratio, weight to volume), and kept in water bath at 100°C for 6 hr, repeatedly for five times. The resulting brownish and dark extracts were filtered and then stored in fridge in dark place [20].

Human lymphocyte culture:

Duplicate peripheral blood cultures were prepared according to Carballo *et al*. [21]. Briefly, heparinized blood samples (0.5 ml), were obtained from healthy donors (non-smoker females, age range 20-25) and were placed in a sterile culture bottle containing 7 ml of RPMI-1640 medium, supplemented with fetal calf serum (1.5 ml), antibiotic–antimycotic mixture (1.0 ml) and phytohaemagglutinin (0.1 ml). The culture bottles were kept in an incubator at 37 °C for 24 hr.

Chromosomal aberrations analysis:

For CA analysis, after 24 hr, 10 µM of Ethinylestradiol treatment (dissolved in dimethylsulphoxide, 5 µl/ml) was given separately with $1.075x10^{-4}$, $2.125x10^{-4}$, $3.15x10^{-4}$ and $4.17x10^{-4}$ g/ml of plant extract, and kept for additional 48 hr of incubation at 37 °C. The untreated control was also kept. After 47 hr, 0.2 ml of colchicine (0.2 µg/ml) was added to each culture vial. Cells were centrifuged at 1000 rpm for 10 min.

The supernatant was removed and 5 ml of pre-warmed (37°C) 0.075 M KCl hypotonic solution was added. Cells were resuspended and incubated at 37°C for 20 min.. After hypotonic treatment the cultures were centrifuged and the supernatant was removed, cells were fixed by adding chilled fixative (Methanol: Acetic acid; 3:1). The fixative was removed by centrifugation and the procedure was repeated twice. The slides were prepared by air drying method and stained with Giemsa stain (3%) in phosphate buffer (pH 6.8) for 20 min. [22]. Later the slides were screened for chromosomal aberrations according to the standard protocol of Savage [23]. For each concentration of treated and control groups 200 metaphases were scored.

Blast Index Assay (BI):

The blast index was calculated as the number of blast cells per 1000 cells analyzed per culture for each treatment. As the following equation:

$$\textbf{BI= no. of the blast cells/total no. of cells ×100}$$

Mitotic index assay (MI):

The mitotic index was calculated as the number of metaphases per 1000 cells analyzed per culture for each treatment. As the following equation:

$$\textbf{MI= no. of the dividing cells/ total no. of the cells ×100}$$

Sister chromatid exchange analysis (SCE):

For sister chromatid exchange analysis, bromodeoxyuridine (10 µg/ml) was added at the beginning of the culture. After 24 hr of incubation, 20 µM of EE (dissolved in Dimethylsulphoxide, 5 µl/ml) treatment was given separately with $1.075x10^{-4}$, $2.125x10^{-4}$, $3.15x10^{-4}$ and $4.17x10^{-4}$ g/ml of pits extract and kept for another 48 hr in an incubator. Mitotic arrest was performed by adding 0.2 ml of colchicine (0.2 µg/ml). Hypotonic treatment and fixation were performed in the same way as for the chromosomal aberration analysis. The sister chromatid exchange average was taken from an analysis of metaphase during second cycle of division [24].

Replication index (RI):

At least 200 metaphases for each treatment were scored to calculate the replication index (RI) by the formula:

$$\textbf{RI= (M1 + 2M2 + 3M3)/100}$$

Where M1, M2 and M3 represent the proportion of the first, second and third metaphases, respectively [25].

Statistical analysis:

Student t-test was used for analysis of CAs, MI and SCEs while chi-square test (χ^2) test was used for the analysis of RI [26].

Results

The results on the incidence of chromosomal aberrations in *in vitro* lymphocytes after treatment with EE and various concentrations of DPE individually and EE primed with DPE are shown in the Table-1.

The results indicate a significant increase of CA by EE compared with the untreated. A significant dose-dependent decrease in number of CA was observed when EE was treated separately with different dosages of DPE (Table 1). Maximum decrease in CA was found at the higher dose of DPE rather than with the lower doses. The decrease in CA was statistically significant ($p<0.05$) for different concentrations of DPE with EE treated groups in comparison to EE alone treated lymphocytes. For DPE alone treated group, the total number of aberrations did not show any significance level of changes. For the EE alone treatment, the total number of aberrations scored 13 which decreased to 2 when treated with both EE and 4.17×10^{-4} g/ml of DPE. This decrease in CA was statistically significant.

Table 1-Effect of different concentrations of date pits extract on CA induced by ethinylestradiol in the cultured human lymphocytes

Type of treatment	Chromatid aberrations			Isochromatid aberrations		Total number of aberration	Number of polyploidy cells
	Gaps	Breaks	Acentric fragments	Gaps	Breaks		
EE (M)							
10	6	7	1	2	3	13[a]	1
EE(M)+DPE(g/mL)							
$10+1.075x10^{-4}$	5	5	1	1	2	8[b]	1
$10+2.125x10^{-4}$	4	3	1	1	1	6[b]	0
$10+3.15x10^{-4}$	2	2	0	0	1	3[b]	0
$10+4.17x10^{-4}$	2	1	0	0	0	2[b]	0
DPE (g/mL)							
$1.075x10^{-4}$	1	1	0	0	0	1	0
$2.125x10^{-4}$	1	1	0	1	0	1	0
$3.15x10^{-4}$	2	1	1	1	0	2	0
$4.17x10^{-4}$	2	1	1	1	0	2	0
Untreated (control)	1	1	0	1	0	1	0

([a]) Significant with respect to untreated (P<0.005); ([b]) Significant with respect to CPA (P<0.005); EE, ethinylestradiol; DPE, date pits extract.

The induction of SCE by EE was decreased at every dose of extract given with EE Table-2. The results also indicate a significant decrease in BI, MI and RI by EE in lymphocytes cultures Table-2. However, a significant dose-dependent increase was observed in BI, MI and RI, when treated with different dosages of the extract Table-2.

Table 2- Effect of date pits extract on BI, MI, RI and SCE induced by ethinylestradiol in the cultured human lymphocytes

Type of treatment	BI% (mean±S.E.)	MI% (mean±S.E.)	Cell Cycle % (mean)			RI% (mean+S.E.)	SCE % (mean+S.E.)
			M1	M2	M3		
EE (M)							
10	11.22±0.08[a]	0.92±0.15[a]	41	49	4	1.51±0.23[a]	6.01±0.40[a]
EE(M)+DPE (g/mL)							
$10+1.075x10^{-4}$	18.75±0.15[b]	1.75±0.46[b]	36	54	7	1.65±0.15[b]	4.32±0.32[b]
$10+2.125x10^{-4}$	24.30±0.05[b]	2.86±0.19[b]	32	58	9	1.75±0.42[b]	3.62±0.30[b]
$10+3.15x10^{-4}$	31.33±0.12[b]	2.74±0.04[b]	30	59	9	1.75±0.09[b]	2.86±0.19[b]
$10+4.17x10^{-4}$	37.85±0.30[b]	3.11±0.10[b]	23	64	12	1.87±0.51[b]	2.44±0.15[b]
DPE (g/mL)							
$1.075x10^{-4}$	38.50±0.50	3.31±0.15	23	65	13	1.92±0.13	1.78±0.13
$2.125x10^{-4}$	36.66±0.07	3.22±0.02	25	64	13	1.92±0.60	1.97±0.19
$3.15x10^{-4}$	35.40±0.13	3.18±0.32	25	62	10	1.79±0.28	2.31±0.27
$4.17x10^{-4}$	35.95±0.22	2.91±0.01	27	61	9	1.76±0.57	2.74±0.18
Untreated (control)	38.72±0.18	3.35±0.12	19	67	14	1.95±0.80	1.33±0.12

([a]) Significant with respect to untreated (P<0.05); ([b]) Significant with respect to CPA (P<0.05); EE, ethinylestradiol; DPE, date pits extract.

Discussion

The parameters used in this study to evaluate the genotoxic effects on cultured human lymphocytes were: CA analysis, BI, MI, RI and SCE. The CA analysis is one of the widely used parameters for testing the protective effects of natural compounds on the drug and chemical induced toxicity. The modulatory effect of natural compounds on the CA induced by various kinds of chemicals and drugs is well established [27-30]. The MI, RI and SCE have been commonly used to evaluate cytogenetic responses to chemical exposure. In a recent study this parameter has been used for testing modulatory effects of allicin and L–ascorbic acid on the chlormadinone acetate induced genotoxicity [31].

This study reveals that ethinylestradiol at 10µM was genotoxic on cultured human lymphocytes. A previous study with ethinylestradiol has shown that the metabolic activation and possible conversion of ethinylestradiol to a reactive species is responsible for the genotoxicity [15].

Medicinal plants and their products have been used for centuries to cure various ailments [32]. Many plant products protect against xenobiotics either by inducing detoxifying enzymes or by inhibiting oxidative enzymes [33].

The results of the present study reveal that the PDE is potent enough to reduce the genotoxic effects of ethinylestradiol at all the selected doses. The selected doses of PDE didn't induce any significant difference in the percentage of all tested assays as compared to control, suggesting absence of its genotoxic effect. Similar findings were obtained in our previous study which has shown that the crude extracts of fruits and pits of date palm exerted a potent anticancerous effect toward some cancer cell lines *in vitro*, and antitumor effect toward transplanted mammary adenocarcinoma in mice [34]. The verification of the possible mutagenic and/or antimutagenic effects of medicinal plants, infusions or extracts is an important factor in such studies. Some plants may possess substances that can modulate the genotoxicity of the other compounds [35]. The protective effect of PDE in the present study i.e. significant reduction in chromosomal aberrations and sister chromatid exchanges or increase in the BI, MI and RI may be due to the direct action of the compounds present in the extract on ethinylestradiol by inactivating it enzymatically or chemically.

The compounds present in the extract may also scavenge electrophiles or nucleophiles [36]. They may also enhance the DNA repair system or DNA synthesis or even may prevent the bioactivation of certain chemicals [37]. The antigenotoxic potential of plant extracts have been attributed to their total phenolic content [36]. Medicinal herbs contain complex mixtures of thousands of compounds that can exert their antioxidant and free radical scavenging effects either separately or in synergistic ways [38]. Identification and characterization of these active principles in the plant extract may lead to strategies to reduce the risk for developing cancer in humans [39]. A study on oral contraceptives and liver cancer has revealed that the oral contraceptives may enhance the risk of liver carcinomas [40]. The present study shows that the PDE reduced the genotoxic effects of ethinylestradiol, and hence suggests the possibility of having lower risk of carcinomas in the patients undergoing ethinylestradiol therapy.

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