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The Role of Oily and Methanolic Extracts of *Phoenix Dactylifera* Leaves in Ameliorating CCl_4 Cytotoxicity in Male Rats

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A B S T R A C T

The study was performed to investigate the protective effect of different methanolic and oily extracts of leave and dry date of *Phoenix dactylifera* against oxidative stress induced by CCl_4 on 49 Sprague-Dawley male rats weighed 175-200 g and aged 6-8 months. The animals were equally divided into 7 groups and assigned as follows: G1, administered 0.1 mL distilled water orally and considered control negative group (C^{-ve}); G2, administered 0.1 mL/100 g BW corn oil (CrO); and G3 administered 100 mg/kg BW CCl_4 orally for induction oxidative stress and considered control positive group (C^{+ve}). The other four groups were initially administered 100 mg/kg BW CCl_4 for oxidative stress induction and treated for two months as follows: G4, treated orally by 100 mg/kg BW of date methanolic extract (DME); G5, treated orally by 150 mg/kg BW of leaves methanolic extract (LME); G6, treated by 250 mg/kg BW date oily extract (DOE); while G7, treated by 250 mg/kg BW leaves oily extract (LOE). At the end of two months experiment, the animals were scarified, and their femurs removed for cytogenetic examination. results showed that only CCl_4 group had significant increase ($P< 0.05$) in mitotic index compared to negative control and all treated groups. CCl_4 group also recorded clear increasing in percentage of chromosome aberrations including diverse types in bone marrow cell compared to rat groups treated by date and leaves methanolic and oily extracts and negative control groups. It could be concluded that the treatment with different palm date and leaves extracts failed to overcome the genotoxic effect of CCl_4 completely. Possibly, because CCl_4 dosed for extended period (2 months) might cause extensive cell and genetic damages could be opposed antioxidants presented in the different palm extracts recording some but lesser chromosomal aberration compared to that CCl_4 treated group.

Keywords: phoenix dactylifera, leave, dry date, oxidative stress, antioxidant, cytogenetic

INTRODUCTION

Most of mutagenic and carcinogen agents display their destructive effects through free radicals including reactive oxygen's species (ROS) (1). The date palm (*Phoenix dactylifera L.*) is one of oldest cultivated plants of humankind used as food for six thousand years. There are more than two hundred varieties of dates

available worldwide especially in Iraq. Date palm serves as a healthy food source as it is rich in carbohydrates and even became a part of Arabian diet. Aside from a common food source, date palm fruits have been used traditionally to treat distinct types of ailments and it has been regarded that consumption of the fruit is good for health (2). Moreover, recent studies have shown that date fruits are an excellent source of phenolics and therefore possess an extremely high antioxidant capacity. Dates have potent anthocyanins, carotenoids, and phenolics compounds (protocatechuic, p-hydroxy benzoic, vanillic, syringic, caffeic, coumaric, ferulic, hydroxy benzoic, mainly cinnamic acids) and flavonoids (flavones, flavanols and flavanones) (3). It is also scientifically proved to possess a variety of pharmacological activities which indicate its usefulness in various kinds of diseases and disorders. It possesses anticancer, antimutagenic, antihyperlipidemic, nephroprotective, and *in vivo* antiviral activities, and the ability to increase the concentration of testosterone, follicle stimulating hormone (FSH) and luteininzing hormone (LH). Many researchers have also documented the antioxidant property of *Phoenix dactylifera* (4). Therefore, this study designated to investigate the cytogenetic effects which play a key role in cancer (genetic damages and changes in DNA sequences and genes mutations and chromosomal structure) of different palm extracts against oxidative stress induced by administration CCl4 to male.

MATERIALS AND METHODS

Collection of Plant Materials and Extraction

Phoenix dactylifera fresh leaves and dry fruits (Al-zahdy) were obtained from Kufa cultivar, Iraq during October 2014. The leaves were washed thoroughly in running tap water, then dried for four weeks at room temperature and blended by mechanical blender, while dry date was cleaned, seeds have been removed and the edible part of date was dried at room temperature before grinding with a meat grinder to produce date paste.

Methanolic extraction of date paste or leaves powder sample were added to 500 ml of Methanol 70% mixed thoroughly by magnetic stirrer for 24 hours according to method (5).

Oily extraction for leaves by Soxhlet apparatus according to Charef, *et al,* (2008) (6), while dry date oily extract according to Soxhlet organic extraction method (7) using hexane and modified by adding methanol to hexane to increase the extract yield of oil by more than double

Experimental Design

This study is undertaken under the approval of the scientific committee of the department of physiology, biochemistry and pharmacology, College of Veterinary Medicine, University of Baghdad. General ethical standards of the animal welfare have been considered.

This experiment was designed and performed on 49 Sprague-Dawley male rats weighed 175-200 g and aged 6-8 month; these animals were purchased from the animal house, College of Veterinary Medicine, University of Baghdad. The animals procured in the air-conditioned room under ideal environment of temperature 25±1° C and dark/light cycle 12/12 hours and left for two weeks for acclimatization before starting the experiment. The animals were equally divided into 7 groups and assigned as, G1 administered 0.1 mL distilled water (DW) orally and considered as a control negative (C^{-ve}), G2 administered 0.1 mL/100 g BW corn oil (CrO), G3 administered 100 mg/kg BW CCl_4 orally for induction oxidative stress and considered as a control positive (C^{+ve}). The other four groups were initially administered 100 mg/kg BW CCl_4 for induction oxidative stress and treated for two months as follows: G4; treated orally by 100 mg/kg BW of date methanolic extract (DME); G5, treated orally by 150 mg/kg BW of leaves methanolic extract (LME); G6, treated by 250 mg/kg BW date oily extract (DOE); while G7, treated by 250 mg/kg.BW leaves oily extract (LOE).

All the dosed materials, drugs and plants extracts were administered as 0.1m/100g .BW of the rat by fitting the solutions concentrations. At the end of the experiment, all animals have been scarified and their femurs were removed for cytogenetic examination.

Cytogenetic Study

Chemicals was used in this study included, Phosphate Buffer solution (PBS) purchased from Chemical point-Germany, has been prepared in laboratory by dissolving 9.86 g of phosphate buffer powder in one litter of distilled water and PH was adjusted to 7.2 using PH meter (Toledo-Switzerland) and used for harvesting bone marrow stem cells. Hypotonic solution of 0.075 prepared by dissolving 1.1175 g of KCL (BDH-company-England) powder in distilled water up to 200 ml for swelling of the stem cells. The cholchicine solution was prepared by dissolving colchicine tablet 1 mg (Mayoly-Spindler-France) in 20 ml D.W. which was used for stopping cell cycle division. Fixative solution consisted of 3 parts of absolute methanol (Scharlau-Spain) and 1 part of glacial acetic acid (BDH-company-England) and used for fixation of stem cells permanently. Giemsa stain stock solution prepared by dissolving 2 g of Giemsa stain powder (Vaccine and Sera institute-Iraq) in 100 ml absolute methanol and mixed well for 3 days by magnetic stirrer (ADAM-India), while the Giemsa working solution was prepared as 1:4 Giemsa stock solution: DW for staining the bone marrow (stem cells) smears.

Parameters

Mitotic index in bone marrow was calculated according to (8) and calculated according to the equation (MI=Number of cell at metaphase/ Total number of cells (1000) ×100).

Chromosamal aberration was evaluated according to the method of (11), briefly by harvesting of bone marrow (stem cells) by PBS, stopping the cell cycle division by cholchicine, swelling the cells by hypotonic solution of KCL, preserving the stem cells in fixative solution, dropping the stem cells from the height 1meter above the slide and finally staining the bone marrow smears by Giemsa stain.

The metaphases were analyzed for the numbers and types of chromosome aberrations that were classified to the chromosomal damages observed in this study which were in the form of fragmented chromosome (FC), acentric chromosome, ring chromosome (RC), elongated chromosome (EC), chromatid gaps (Ga), chromatiddeletions (Cd), and aneuploid. In each slide, 100 mitotic cells were counted to determine the chromosome aberrations.

RESULTS

Mitotic Index (MI)

Cytological examination revealed that CCl4 was found to be effective in increasing cell proliferation (estimated as mitotic index). The results presented in Table (1) showed that the analysis of mitotic activity after treatment with carbon tetrachloride alone showed significantly increased P< 0.05 in MI (Figure-1) compared to negative control (C $^{-ve}$ DW). Treatment with CCl4+ methanolic extract of date and leaves at dose 100, 150 mg/kg respectively were showed significant decrease in MI from that of the CCl4 alone and significantly (P<0.05) increase when compared to control distilled water group (C $^{-ve}$ DW) (Figure 4), while the groups treated with CCl_4 +oily extracts of date and leaves at dose 250 mg/kg showed significant decrease (P<0.05) compared to CCl_4 group but without significant difference from methanolic date extract (Figure 3).

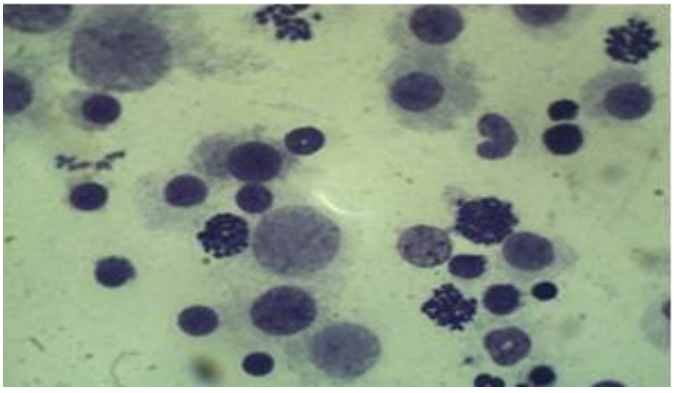

Figure 1. Shows mitotic cell in positive control group treated with CCl_4. 40×

Table 1. Mitotic index of bone marrow cell in rats treated with date and leaves alcoholic and oily extracts with CCl_4 compare with control groups

Groups	Mitotic Index±mean
DME	1.4±0.30 ^{B}
LME	1.9±0.10 ^{B}
DOE	2.1±0.06 ^{B}
LOE	2.3±0.10 ^{B}
C^{+ve} CCl_4	5.1±0.50 ^{A}
C^{-ve} CrO	1.9±0.07 ^{D}
C^{-ve} DW	0.9±0.20 ^{C}

LSD=0.81. $^{A-C}$Different capital letters refers to significant results between groups (P≤0.05). **DME**, date methanolic extract= treated orally by 100 mg/kg BW of DME; **LME**, leaves methanolic extract= treated orally by 150 mg/kg BW of LME; **DOE**, date oily extract=treated by 250 mg/kg BW DOE; **LOE**, leaves oily extract=treated by 250 mg/kg BW LOE; **C^{+ve}**, control positive=administered 100 mg/kg BW CCl_4 orally for induction oxidative stress; **CrO**, corn oil=administered 0.1 mL/100 g BW; **C^{-ve}**, control negative=administered 0.1 mL distilled water orally

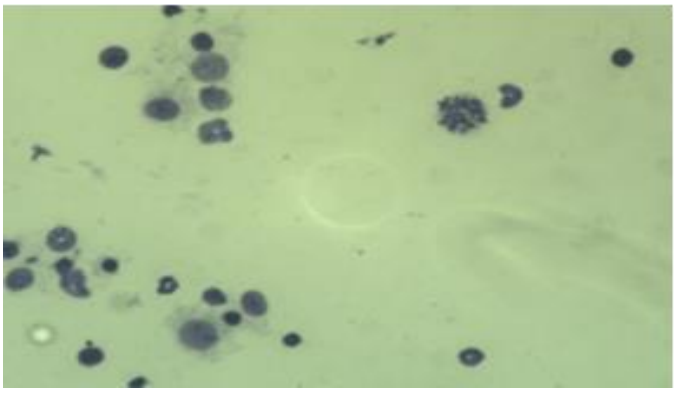

Figure 2. shows mitotic cell in negative control group with distilled water. 400×

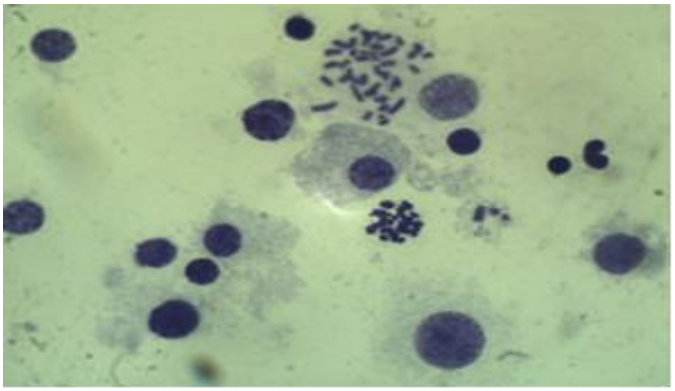

Figure 3. Shows mitotic cell in group treated with date oily extract+CCl_4. 400×

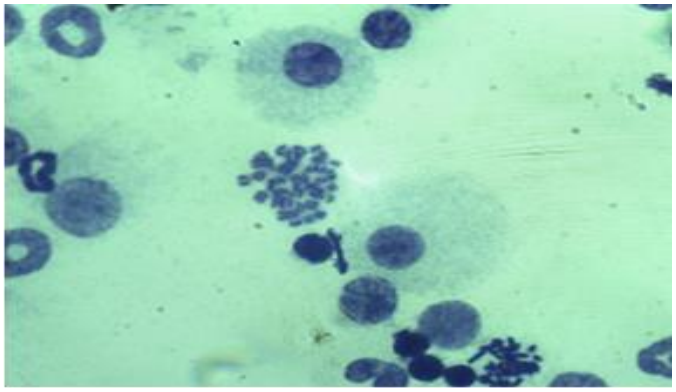

Figure 4. Shows mitotic cell in group treated with leaves methanol extract+CCl_4. 400×

Chromosomal Aberration

Various types of structural chromosomal aberrations (Figure 5) were observed after the administration of carbon tetrachloride alone or plus date and leaves of methanolic and oily extracts compared to the negative control groups included fragmented chromosome (FC), a centric chromosome (AC), ring chromosome (RC), elongated chromosome (EC), chromatid gaps (Ga), chromatid deletions (Cd), segmental deletion (SD)and aneuploid as shown in figures (5). CCl4 group recorded clear increasing in percentage of chromosome aberrations compared to rat groups treated by date and leaves methanolic and oily extracts and negative control groups while the groups treated by date and leave oily extracts still recorded more cytogenetic changes compared to oil and distilled water control groups (Table 2).

Table 2. Percentage of some chromosomal aberrations of bone marrow cells of experimental group

Groups n=5	Chromosomal Lesion							Total
	Fragmented chromosome (%)	Chromatid deletion (%)	Ring chromosome (%)	Gap chromatid (%)	Stick chromosome (%)	Elongated chromosome (%)	Aneuploidy (%)	
C-ve DW	3	0.3	0	0	0	0	0	0.033
C-ve CorO	10	0.6	4.3	3	0	3	1.4	2.83
C+ve	25	15	8.9	10	7	6	12.6	0.845
DME	17	7.2	2.5	5.1	4.8	2.5	2.1	0.412
LME	18.2	9.3	5	6	4	4.3	3	0.498
DOE	18.4	8.5	5.7	6.5	4.8	3.6	4.3	0.518
LOE	19.1	10.9	5.3	6.8	5.2	4.4	4.9	0.566

C-ve, control negative=administered 0.1 mL distilled water orally; **CrO**, corn oil=administered 0.1 mL/100 g BW; **C+ve**, control positive=administered 100 mg/kg BW CCl_4 orally for induction oxidative stress; **DME**, date methanolic extract= treated orally by 100 mg/kg BW of DME; **LME**, leaves methanolic extract= treated orally by 150 mg/kg BW of LME; **DOE**, date oily extract=treated by 250 mg/kg BW DOE; **LOE**, leaves oily extract=treated by 250 mg/kg BW LOE

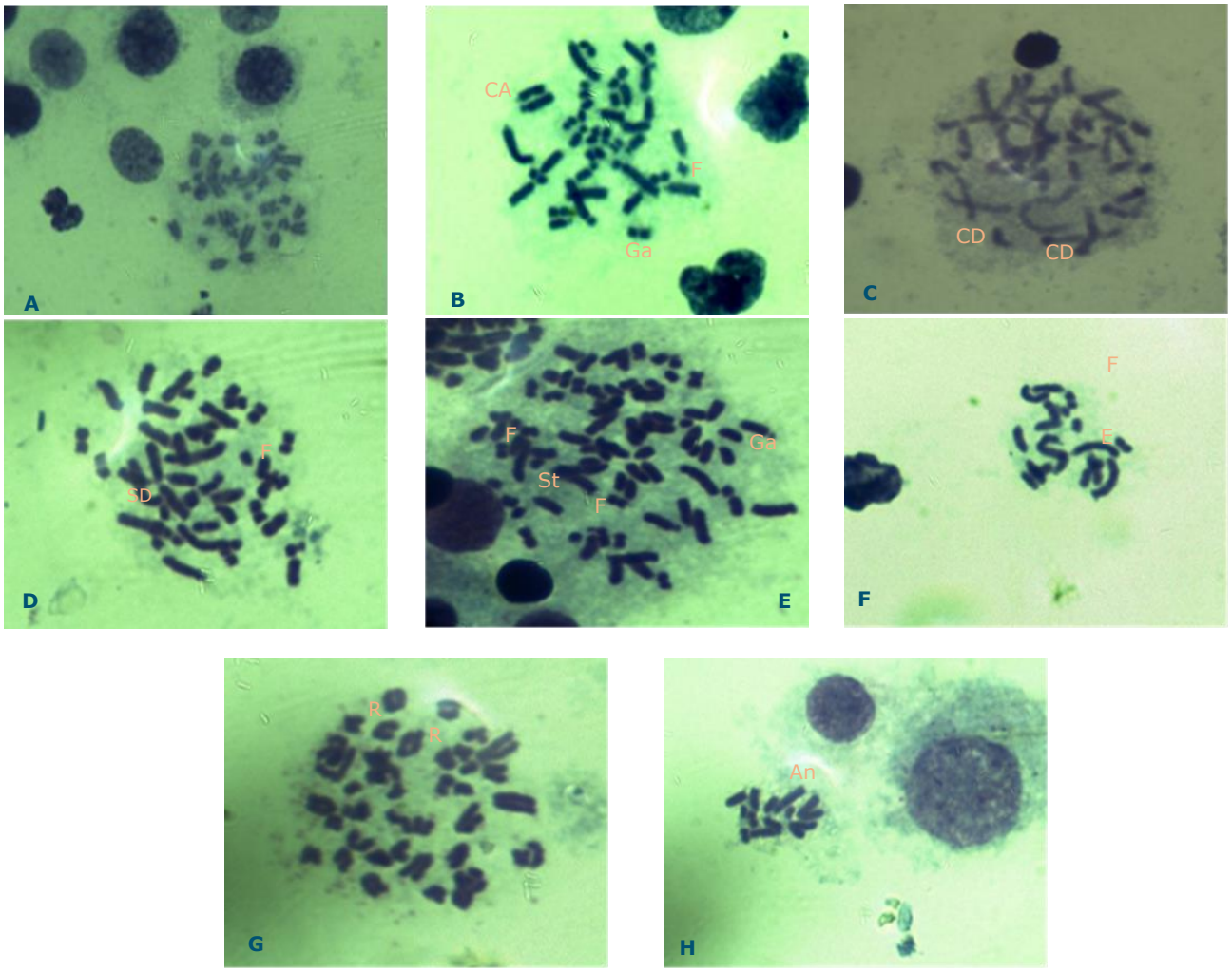

Figure 5. The different chromosomal aberrations from bone marrow cells of rats from different experimental groups. **(A)** normal chromosome. **(B) acentric** chromosome (AC), fragmented chromosome (FC), chromatid gap (Ga). **(C)** chromatid deletion(CD), chromosome deletion(CD). **(D)** segmental deletion(SD), chromosome fragment (F) , chromatid gap (Ga). **(E)** stickiness (St), chromosome fragment (F), chromosome gap (Ga). **(F)** chromosome elongation (E), chromosome fragment (F). **(G)** ring chromosome (R). **(H)** aneupoloid (An)**(**Giemsa stain, 100×).

DISCUSSION

The results of present cytogenetic study in bone marrow cell of all subchronically treated experimental groups rat treated with CCl4 showed highly extensive genotoxic effects possibly because of high exposure for two months effects causing two types of chromosomal mutation one is clastogenesis with structural chromosomal or chromatid damages manifested by (chromosome fragment, chromatid deletion, ring chromosome, elongated chromosome, chromosome gap, stick chromosome and acentric chromosome). The second type is aneuploidy that is considered (numerical) chromosome change.

The results were obtained by many studies (12) and (13) and (14), showed that CCl4 treatment induced different types of chromosome aberration (CA) with increased of the number of dividing cells.

A possible mechanism for CCl_4 induced chromosomal aberration involved in the formation of free radicals, either via auto-oxidation or by enzyme- catalyzed oxidation of organic compounds in CCL4. These free radicals could react with lipids and may lead to lipid peroxidation of cell membrane in tissues causing the breakage of the DNA chain by oxidizing the base in DNA and covalent binding between the product of lipid peroxidation and DNA. They could also react with proteins, affecting the structures and functions of enzymes, and alter the membrane properties. In addition, the free radicals could also attack nucleic acids, especially some spots in purine and pyrimidine, resulting in base substitution and breakage of DNA, and eventually inducing mutation (12). The noticed chromosomal aberration mainly in CCl4 group and to lesser extent in palm oily and methanolic extracts was: Ancentric chromosome is an aberrant chromosome that lacks a centromere. Ring chromosome forms when a portion of a chromosome is broken off and formed a circle or ring. They are basically deficiencies and therefore render the carrier liable to the usual consequences of a missing genetic material (12) or induction of ring chromosomes suggesting the possibility of two breaks that occur in the same chromosome or may result from telomeric losses. According to Hall and Garcia (14), presence of ring chromosomes is a condition which is highly lethal to the cell.

Stickiness of chromosomes is one of the major abnormalities noted in the present study. It could be observed different degrees of stickiness in all the treatments. Stickiness might be caused by the physical adhesion of chromosomal proteins or due to the disturbances in the nucleic acid metabolism of the cell or the dissolution of protein covering of DNA in chromosomes (15).

Aneuploid cells noted in the treatments might be due to the occurrence of multipolar mitosis or lagging chromosomes producing two aneuploid daughter cells.

Chromosome fragments are signs of extreme lethal clastogenic effects resulting from chromosome and chromatid breaks. Fragments may arise due to stretching of chromosomes at metaphase followed by breakage at these fragile sites (12).

While all palm extracts (alcoholic and oil) recorded less but still present some genotoxic effect compared to CCl4 and control groups. These results are in agreement with those obtained by Gabal et al. (2007) who recorded an increase in the frequency of chromosomal aberrations in both bone marrow cells of male mice treated with CCl_4 (16).

Alghamdy et al. (2013) studied the protective effect of date extract against genotoxic effect performed by bone marrow cell, and recorded analysis of chromosomal aberrations in the efficacy of date extracts in reducing the frequency of different types of chromosomal aberrations induced in bone marrow cells in particular aneuploidy and ring chromosome (17). This finding suggested that date extract has antimutagenic effect and protects the cells from the mutagens which it contains highly quantity of protein and fat such as oleic, linoleic, palmitic, myristic and stearic acid when compared to date flesh. It is also high in dietary fibers, phenolics and antioxidants. It is assuming that cumulatively the presence of all these components in the date may have responsible for reduction of DNA binding and methylation with CCl4 (18). The current findings on DNA damage are in agreement with Aboul-Enein, et al. (2014) who found that administrations of CCl4 to rats caused significant increase of DNA damage as compared to normal control.

The current cytogenetic results showed extensive genotoxic effect in CCl4 group with fewer effects recorded in bone marrows of date and leaves extracts treated groups. All of these types indicated that the genotoxic effect of CCl4 was so extensive that possibly causing nonsense mutation with complete loss of proper genetic function that may give rise to disturbance in their cell cycles division promoting for enhancement of cell division with higher opportunity of development of cancer. The increase in mitotic index of CCl4 treated group was only indicative of such conclusion. This sort genotoxic effect was mostly seen in progressive stage of cancer with karyotypic instability leading to development of cancer or genetic disorder (18). On the other hand, palm extract of fruits and leaves caused significant reduction in the mitotic index as well as less extensive genotoxic effect as indicative of partial protection against CCl4 cytogenetic effect.

Treatment with different palm date and leaves extracts did not succeed to overcome completely the genotoxic effect of CCl4. Possibly, because CCl4 dose for long period (2 month) might cause extensive cell and genetic damages that cannot be opposed antioxidants presented in the different palm extracts recording some, but lesser chromosomal aberration compared with that CCl4 treated group.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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تأثير الحماية الخلوية الجينية للمستخلصات الدهنية والكحولية للتمر الزهدي *Phoenix dactylifera* الجاف والسعف في ذكور الجرذان

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الخلاصة

انجزت الدراسة لتحقيق تاثير المستخلصات الكحولية والدهنية للتمر الجاف والسعف للنخيل العراقي من صنف الزهدي فيونكس دكتي لايفيرا *phoenix dactylifera* ضد الاكسدة المستحدثة بواسطة تجريع رابع كلوريد الكربون على تسعة واربعون من ذكور الجرذ Sprague-Dawley اوزانهم 175-200 غم وبعمر 6-8 اشهر. قسمت بالتساوي الى سبعة مجاميع خصص G1مجموعة الاولى السيطرة السلبية اعطيت 0.1 مل ماء مقطر , G2 مجموعة الثانية اعطيت 0.1 مل / 100 غم من وزن الجسم زيت الذرة G3 مجموعة الثالثة السيطرة الايجابية اعطيت 100ملغم / كغم رابع كلوريد الكربون فمويا لأحداث الاكسدة. المجاميع الاربعة الاخرى اعطيت يوميا عن طريق الفم 100ملغم / كغم رابع كلوريد الكربون لاحداث الاكسدة والعلاج لمدة شهرين فمويا. G4 بالمستخلص الكحولي لتمر الجاف 100 ملغم /كغم , G5 مستخلص الكحولي لاوراق النخلة 150 ملغم / كغم , G6 مجموعة علاج بالمستخلص الدهني لتمر الجاف بجرعة 250 ملغم / كغم بينما G7 بالمستخلص الزيتي للأوراق بجرعة 250 ملغم / كغم . بعد مرور شهرين قتلت الحيوانات وتم اجراء جمع وتحضير نخاع العظم المنزوع لغرض اجراء الفحص الخلوي الجيني لتاثير المستخلصات المضادة للتاثيرات الجينية المستحدثة برابع كلوريد الكاربون في جميع مجاميع التجربة وذلك بدراسة معامل الانقسام الكروموسومي ونسب تشوهات الكروموسومية. اظهرت النتائج الدراسة الجينية في خلايا نخاع عظام مجاميع التجربة عدم وجود اي اختلاف معنوي في معامل الانقسام الخلوي لكل مجاميع المعالجة بالمستخلص بالمقارنة مع مجموعة السيطرة الزيتية والمائية بينما اظهرت مجموعة المعالجة برابع كلوريد الكاربون زيادة معنوية عالية معنوية بمعامل الانقسام بالمقارنة مع بقية المجاميع اما نتائج فحص التشوهات الكروموسومية اظهرت مجموعة المعالجة بـ CCl_4 زيادة معنوية بنسبة التشوهات الكروسومية التركيبة مقارنة بالمجاميع العلاجية للمستخلصات ومجاميع السيطرة .تبين من هذه الدراسة بان المستخلصات الميثانولية والزيتية للتمر الزهدي وسعفه ليس لها القدرة الكاملة على التغلب على التأثيرات الجينية المحدثة بواسطة رابع كلوريد الكربون وقد يكون سبب ذلك هو فترة التعرض الطويلة نسبيا (شهرين) لرابع كلوريد الكربون والتي ادت الى تاثير جيني كبير

الكلمات المفتاحية: : نخيل فيونكس دكتي لايفيرا، تمر الجاف، السعف، جهد الاكسدة، مضاد الاكسدة، الدراسة الجينية