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ANTICLASTOGENIC ACTIVITY OF THE DRUG SILYMARIN

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ABSTRACT

This work aims at investigating the anticlastogenic activity of the drug silymarin. To achieve such a purpose four doses of the drug i.e., 2, 4, 8, and 20 mg/ kg. b. wt. were tested employing mice genome (*Mus musculus*, 2n= 40).

The following genotoxic bioassays were used: 1- Estimation of cell proliferation. 2- Analysis of chromosomal abnormalities in mice bone marrow cells. 3- *In vivo* induction of sister chromatid exchanges, 4- Micronucleus test, and 5- Analysis of primary spermatocytes. Cyclophosphamide (Indoxan) was used as a positive control.

The results obtained revealed that silymarin was proven to be capable in causing significant increases in cell proliferation (estimated as mitotic index) of mice bone marrow cells; decreasing in total aberrant metaphases as well as in SCEs *in vivo*. Micronucleated polychromatic erythrocytes and aberrant diakinesis were significantly decreased; giving an evidence that silymarin has a strong anticlastogenic activity upon mice genome in somatic as well as germinal cells.

INTRODUCTION

The increasing exposure of humans to newly synthesized drugs and chemicals has urged the scientists to develop reliable assay systems for detecting whether any of these agents are potential mutagens; carcinogens; and/ or clastogens.

Drugs are normally prescribed by physicians all over the world as a symptomatic treatment for diseases (Seehy & Osman, 1989; and Youssef, 2000). The drug silymarin, the active principle of Mariagon, is of a plant origin (fruit of *silybum marianum*). The mechanism of silymarin action involves membrane stabilization, neutralization of free radicals and immunomodulatory effects. Direct protection of the liver cell can be achieved by its membrane-stabilizing properties, thus re-establishing their ultra-structure and restoring their metabolic, diestive and detoxicating functions to normal. Clinically, it is capable of protecting the liver against harmful and toxic agents. Also, it accelerates the regenerative capacity of the damaged liver cells. These effects are reflected in the form of improvement in general condition, amelioration of digestive disorders, modulation of other signs of liver disease and normalization of liver function tests. Silymarin exerts an anti-oxidant activity that might be one of the important factors in the hepatoprotective action of this product. This anti-oxidant activity is explained by increased activity of both superoxide dismutase and glutathione proxidase thus increasing the "free radical elimination capacity" of the glutathione peroxidase system. Mariagon exerts general supportive and promoting effects on cell metabolism and hence, has an overall protective effect on liver function. It is used for the protection of the liver in cases of intoxication (environmental, drug, diabetic and alcohol induced), Adjuvant to the treatment in chronic liver diseases e.g. fatty liver, liver cirrhosis …etc. and for treatment of male and

female infertility due to hormonal imbalance caused by hepatic dysfunction (Lee et al, 2007)

This investigation was conducted to assess the anticlastogenic activity of this drug.

MATERIALS AND METHODS

The drug was locally purchased and four doses i.e., 2, 4, 8; and 20 mg/ kg. b. wt. were selected and tested. Mice (*Mus musculus*, 2n= 40) were used by employing the follwing assays:

Analysis of chromosome behavior:

Each animal had orally received daily the proper dose for 7 days. The animals were killed by decapitation 24 hr after the last dose. For each treatment, four animals were used. Animals of the control group (4 for each treatment) received equivalent amounts of deionized water. Three hours prior to killing, the animals were injected with 0.6 mg/kg of colchincine.

After killing, the adhering soft tissue and epiphyses of both tibiae were removed. The marrow was aspirated from the bone, transferred to phosphate buffered saline, centrifuged at 1000 rpm for 5 minutes and the pellet resuspended in 0.075 M KCL. Centrifugation was repeated and the pellet was resuspended in fixative (methanol: acetic acid, 3:1). The fixative was changed after 2 hours and the cell suspension was left overnight at 4 C.

Slide preparation and staining

Cells in fixative were dropped on very clean glass slides and air-dried.

Spreads were stained with 10% Giemsa at PH 6.8 for 5 min.

Screening of slides

Slides were coded and scored for chromosomal aberrations e.g. gaps and deletion, fragment, break, stickiness and polyploidy. A mitotic index based on at least 1000 counted cells was recorded. For chromosomal abnormalities, at least 200 metaphase cells per dose were recorded Comparison with control was also statistically tested when needed.

Sister chromatid exchange technique

Experimental design

Typically 2-3 month old mice, 2n = 40 were used. Four animals per dose were used and analysis of at least 25 cells per animal was carried out. Four selected doses were administrated. A dose response curve was established and extended over at least a 10 fold does range and contained four informative doses plus that of the negative control as well as positive group.

Bomodeoxyuridine tablet preparation

Bromodeoxyuridine tablets were prepared as described by Allen *et al*. 1978; Allen, 1982; and Seehy *et al*. 1983 as follow : Bromodexyuridine tablets were prepared by using pellet press (Parr instrument co , Moline, III ., USA) equipped with a 0.178 in diameter punch and die. Approximately, 200 mg of pure Bromodeoxyuridine powder were weight, placed in the die, and pressed. In order to maintain consistent compaction hardness (and thus the dissolution rate) among tablets, utilization of the same personal and die adjustment when pressing the powder was conducted. BrdU tablets were protected from light and stored in a freezer until usage.

BrdU Treatment

The animals were lightly anesthetized by placing it in a closed container with ether unit immobile (about 2 min). After removal from the container, each animal was restrained on its back. A small vial of anesthesia was placed near its nose for use in prolonging the inactive state. The lower lateral region was swabbed with alcohol in order to mat the fur down. Clean scissor or a scalpel was used to make a small (approx. 1 cm) subcutaneous incision. In order to spread open a deeper subcutaneous pocket, forceps were used, and the tablet was inserted. The wound was then closed with 2 - 3 outclip sutures taking full care not to break the tablet and the animal was received the proper doses of the drug 8 hr after BrdU treatment. Each animal was injected intravenously with 20 mg colcemid (0.1 m1) animal, in tail vain) at hr 19 - (following BrdU treatment). Control marrow cells harvested 2 hr later revealed high fraction of metaphases of optimal sister chromatid differentiation after staining.

Marrow cells harvest and slide preparation

The animal was killed by cervical dislocation. Both femurs were immediately removed, and cleaned of extraneous tissues. Bone tips cut away so that a small syringe needle (i.e. 26 gauge) can be inserted and femoral contents were flushed with phosphate buffered saline (8 g NaCl, 0.2 g KCl, 2.17 g $Na_2 HPO_4 + H_2 O_3$ 0.2 g $KH_2 PO_4$, are dissolved in 1 L and PH is adjusted to 7.0) into a small common tube (total cell solution volume of about 8 ml). Cell suspension was centrifuged at 1000 rpm for 5 min. the supernatant was discarded and cell pellet disrupted by flicking the base of the tube. A hypotonic solution of potassium chloride (0.075 M) was added to give a light cloudy solution (about 8 ml), and let stand for 12 min. The cell suspension was centrifuged, and the supernatant was discarded, cell pellets were fixed in a fixative solution (3 parts methanol: 1 part glacial acetic acid) for 10 min. then centrifuged and the supernatant was discarded. Fixation was repeated for 10 min, followed by centrifugation and the supernatant was discarded. Final fixation performed in 4-5 mL fresh fixative. The slides were prepared as follow: 3 drops of freshly fixed cells were added to clean dry side, dropping the cells from about 1-2 ft distances. Cell density was checked through the microscope more drops were added if needed. The slides were then stored protected from light.

Slide staining. Staining was performed by the method of Goto *et al*. (1978).

The slides were stained with 50u /ml of Hoechest 33258 dye in distilled water, PH 7.0 for 10 min (protected from light). The slides were rinsed in water, and covered by a layer of Mc llvaines buffer (add 18 ML OF Solution A (1.92% citric acid) to 82 ml of solution B (2% disodium phosphate) and adjust the PH of 7.0 or 7.5 with further mixing), mounted by cover slip and subjected to light with intensity <= 400 nm, at a distance of about 2 inches for 20 min. During this time, slides were placed on a wormer tray at 50 C. the slides were then rinsed in distilled water and immersed in 4% Giemsa dye, rinsed in water and allowed to dry for subsequent light microscope analysis.

Screening of slides and analysis

Scanning slides for mitotic spreads was conveniently accomplished with a 25 x magnification objective, and analysis was with a 100 x objective. For control of bias, all prepared slides were coded prior to scoring. There are two ways for counting sister chromatid exchange frequencies i.e., (1) from the microscope images of second division cells, (2) the cells may be photographed and SCE frequencies counted from the microscope images. An interstitial exchanged segment was counted to be 2 SCEs.

Usually, wide ranges of SCE values were encountered specially in treated cells, and then the analysis of variance using F- test was applied. To evaluate the differences in mean of SCE frequencies between treated and control groups, Duncan's multiple range test was used (Snedecor, 1958).

Micronucleus Test:

Four mice were used for each dose. Experimental design was done as that described by Brusick (1986).

Bone- marrow smears were made according to Schmid (1975). Staining was carried out according to the method described by Gollapudi and Kamara (1979). The data were analyzed according to Hart and Pederson (1983).

Analysis of mouse primary spermatocytes:

For each dose, four male mice were used. The used procedure follows basically the description given by Oud *et al* (1979) and Adler (1984).

RESULTS

Cell proliferation:

Table (1) shows the mitotic index as an indicator for cell proliferation. It was 8.2% in the negative control, which in positive control it was 4.1% (for 25 mg/ kg. b. wt.), and it was 2.2 (for 5o mg/ kg. b. wt.). As shown in this table mitotic index was increased after treatment with the tested drug to be ranged from 10/3% to 16.8% (2 folds when compared with that of the negative control), giving the first evidence that silymarin drug induced significant increases at the level of all used doses. Treatment with the tested drug in combination with cyclophosphamide (Table, 1), shows that, although mitotic activity was proven to be lower than that obtained after treatment with the

drug alone, however, mitotic activity was found to be either equal to the negative control or higher, giving the second evidence that the drug plays an important role against cyclophosphamide effect.

Table (1): Mitotic activity in mice bone marrow cells after treatment with the tested drug silymarin.

Dose;Mg/ kg.b.wt.	MI ± S.E.	MI ± S.E.	
		Dose + PC1	Dose + PC2
2	10.3 ± 1.2	8.2 ± 1.1	7.1 ± 1.1
4	12.7 ± 1.1	9.4 ± 1.4	8.2 ± 1.2
8	16.2 ± 1.4	11.3 ± 1.7	10.3 ± 1.4
20	16.8 ± 1.6	14.1 ± 2.1	11.4 ± 1.3
NC	8.2 ± 1.1	———	———
PC:			
PC1: 25 mg	4.1 ± 0.4	———	———
PC2 : 50 mg	2.2 ± 0.1	———	———

Chromosomal aberration in bone marrow:

Table (2) illustrates the results obtained after treatment with the different doses of the drug. No increased in aberrations might be detected except that obtained after treatment with cyclophosphamide, giving the third evidence that the drug does not cause chromosomal aberrations. Treatment with silymarin in a combination with cyclophosphamide (Table, 3) revealed that the drug, at the level of this study, was shown to be positive in decreasing different types of chromosomal aberration.

Table (2): Chromosomal abnormalities in mice bone marrow cells after treatment with the tested drug silymarin

Dose, Mg/ kg.b.wt.	Type of aberrations						Total aberrant Metaphase, %
	Stickiness	Gap	Fragment	RCF	Polyploidy	Others	
2	2	—	1	—	——	——	3
4	3	—	——	—	——	———	3
8	2	1	——	—	——	——	3
20	4	—	1	—	——	——	4
NC	3	—	1	—	——	——	4
PC							
PC1: a-25 mg	20	5	6	3	4	2	40
PC2: b-50mg	35	6	8	3	5	3	59

NC: Negative Control **PC: Positive Control**

Table (3): Chromosomal abnormalities in mice bone marrow cells after treatment with a combination of silymarin and cyclophosphamide

Dose+ PC1 or PC2	Type of aberrations						Total aberrant Metaphase
	Stickiness	Gap	Fragment	RCF	Poly ploidy	others	
2mg + PC1	12	2	3	4	5	—	26
4mg + PC1	10	—	3	2	3	1	18
8mg + PC1	10	3	1	2	\	2	19
20mg + PC2	8	1	—	—	—	2	11
2mg + PC2	30	7	8	4	6	1	56
4mg + PC2	32	6	6	2	2	—	48
8mg + PC2	20	2	2	2	—	2	28
20mg + PC2	12	—	—	—	—	—	12

PC1: 25 mg cyclophosphamide / kg.b.wt.
PC2: 50 mg cyclophosphamide / kg.b.wt.

***In vivo* induction of SCEs:**

Table (4) shows the averages of SCEs obtained after treatment with the tested doses of the drug in addition to the negative and positive control groups.

Table (4): In vivo induction of sister chromatid exchanges in mice bone-marrow cells

Dose; mg/ kg. b. wt.	* $\overline{X}$ ± S.E	Range
2	3.8 ± 0.4	2 – 4
4	2.5 ± 0.2	1 – 5
8	2.6 V ± 0.2	2 -5
20	1.2 ± 0.1	0 – 3
NC	3.2 ± 0.3	2 – 5
PC(25 mg)	14.6 ± 1.8	8 - 22

*** Per cell**

The data in table (4) shows that the drug itself is a negative inducer of SCEs, giving evidence that the drug is a negative inducer of primary DNA damage. Analysis of variance showed that there was a significant difference. Duncan's multiple range test (Table, 5) showed that all tested doses were different significantly from the positive control. In addition, comparing the average of SCEs obtained from the negative control and that after treatment with 20 mg/ kg.b.wt. one can conclude that this dose was proven to repair primary DNA damage.

Table (5): Duncan's multiple range test for mean differences of SCEs

Dose; mg/ kg. b.wt.	$\overline{X}$	$\overline{X}$ - X_{20}	$\overline{X}$ - X_4	$\overline{X}$ - X_8	$\overline{X}$ - X_{NC}	$\overline{X}$ - X_2
PC	*14.6	*13.4	*12.1	*12.0	*11.4	*10.8
2	3.8	*2.6	1.3	1.2	0.6	
NC	3.2	*2.0	0.7	0.6		
8	2.6	1.4	0.1			
4	2.5	1.3				
20	1.2					

- **Significant at 0.05 level of probability**

Micronucleus Test:

Table (6) illustrates the data obtained from the analysis of micronucleated polychromatic erythrocytes obtained after treatment. The results clearly show that the tested drug was found to decrease, significantly, the formation of micronucleus in immature red cell, giving an evidence that it plays an important role as anticlastogenic agent.

Table (6): Micronucleated polychromatic erythrocytes in mice bone marrow after treatment with the tested drug

Dose; mg/ kg. b. wt.	Total PCE counted	PCE with micronucleus	%PCE with micronucleus
2mg + PC1	4000	118	2.9*
4mg + PC1	4000	96	2.4*
8mg + PC1	4000	40	1.0*
20mg + PC2	4000	22	0.55*
2mg + PC2	4000	206	5.15*
4mg + PC2	4000	140	3.5*
8mg + PC2	4000	98	2.45*
20mg + PC2	4000	44	1.1*
Negative Control	4000	12	0.3*
PC1	4000	210	5.2*
PC2	4000	318	7.9*

Analysis of Primary spermatocytes:

Table (7) shows the data obtained from the analysis of diakinesis stage after treatment with the different doses of the tested drug. Data show that the drug was found to be anticlastogenic agent in germinal cell of mice.
Figures 1-4 illustrate different types of aberrations induced by cyclophosphamide and the anticlastogenic activity of the tested drug. (Figures 1-4, represent some of the different types of aberrations).

Table (7): Analysis of diakinesis stage after treatment with the tested drug

Dose; mg/ kg. b. wt.	Stickiness	Exchange	Breaks	Translocation	Univalents xy and/ or autosomal	% of total Aberrant diakinesis
2mg + PC1	11	4	3	2	8	28
4mg + PC1	9	1	4	2	6	22
8mg + PC1	2	2	5	3	4	16
20mg + PC2	2	1	2	1	——	6
2mg + PC2	16	4	6	4	10	40
4mg + PC2	10	6	4	6	12	38
8mg + PC2	9	2	5	3	8	26
20mg + PC2	6	2	3	3	4	18
Negative Control	4	——	—	——	——	4
Posative Control						
PC1	20	8	4	2	4	38
PC2	28	12	6	8	6	60

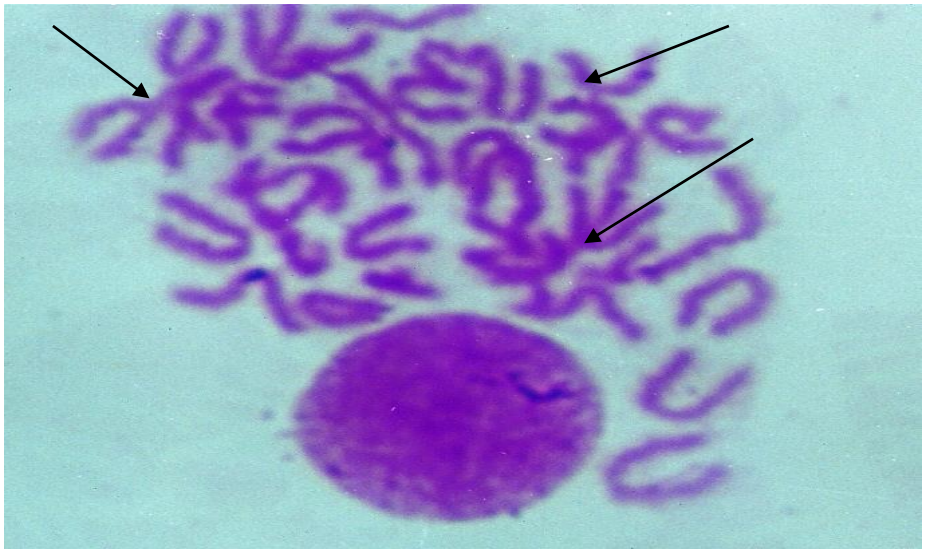

Figure (1): Photomicrograph showing stickiness after treatment with the positive control.

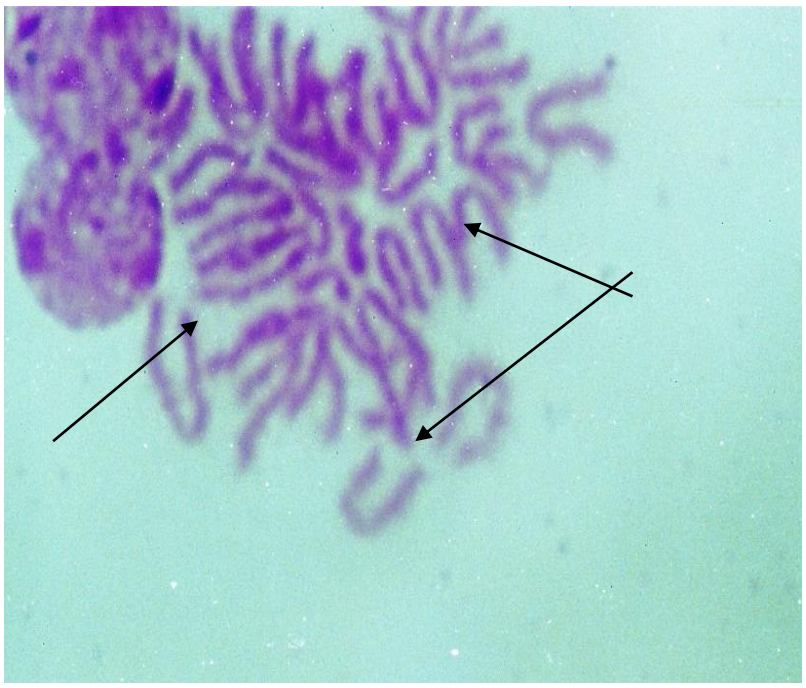

Figure (2): Photomicrograph showing chromatid deletion induced after treatment with the positive control

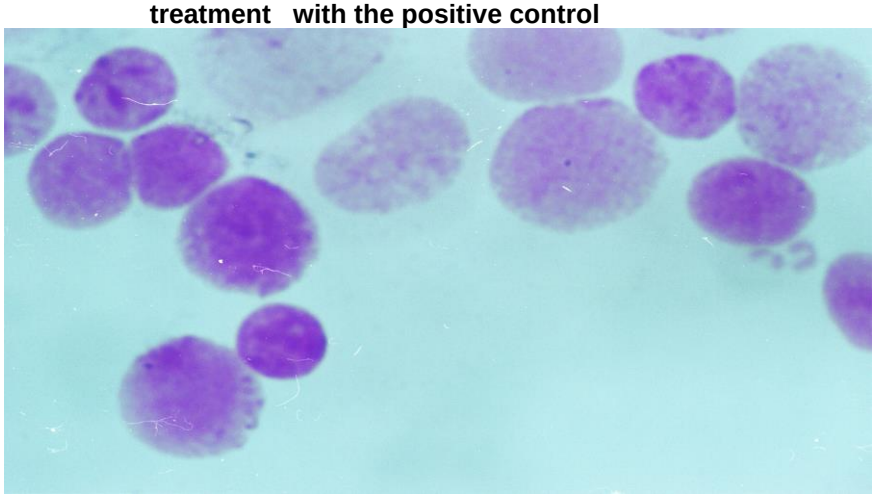

Figure (3): Micronuleated polychromatic erythrocytes in the negative con

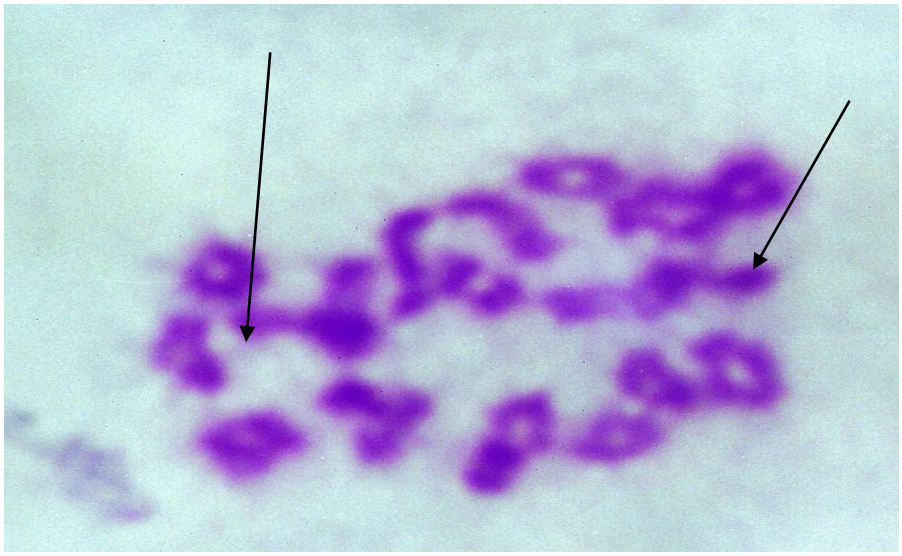

Figure (4): Diakinesis stage showing exchange after treatmeant with the positive control

DISCUSSION

The use of negative and positive control groups is recommended in all mutagenicity tests. According to Preston *et al* (1987), positive controls are included to establish the ability of the analyzers to correctly determine aberrations and to ascertain the expected test- to- test and animal- to- animal variations, and to establish the sensitivity of a particular test. However, cyclophosphamide is a clastogenic agent for various animal species. Chorvatovicora and Sandula (1995) recommended the use of this drug in cytogenetical studies.

As shown in table (5) statistical analysis revealed that a dose level of 20 mg/kg.b.wt. was found to be significantly decreased the frequency of SCEs, giving an evidence that the tested drug has a specific capability to repair DNA lesion.

Several studies have demonstrated that diabetic patients with cirrhosis require insulin treatment because of insulin resistance. As chronic alcoholic liver damage is partly due to the lipoperoxidation of hepatic cell membranes, anti- oxidizing agents may be useful in treating or preventing damage due to free radicals. The aim of this study was to ascertain whether long- term treatment with silymarin is effective in reducing lipoperoxidation and insulin resistance in diabetic patients with cirrhosis.

LE Magazine (1997&2007) reported that there was a significant decrease ($P< 0.01$) in fasting blood glucose levels, mean daily blood glocose level, daily glucosuria and HbA1c levels aleady after 4 months of treatment of treatment in the silymarin group. In addition, there was a significant decrease ($P< 0.01$) in fasting insulin level and mean exogenous insulin requirements in

the treated group, while the untreated group showed a significant increase ($P< 0.05$) in fasting insulin levels and a stabilized insulin need. These finding are consistent with the significant decrease ($P< 0.01$) in basal and glucagon – stimulated C-peptide levels in the treated group and the significant increase in both parameters in the control group. Another interesting finding was the significant decrease ($P< 0.01$) in malondialdehye/ levels observed in the treated group.

These results show that treatment with silymarin may reduce the lipoperoxidation of cell membranes and insulin resistance, significantly decreasing endogenous insulin overproduction and the need for exogenous insulin administration.

The present work revealed that silymarin has anticlastogenic activity upon somatic as well as germinal cells as shown as the analysis of chromosomal aberration; micronucleus test; and analysis of diakinesis stage in mice primary spermatocytes. It has the capability to lower the induction of sister chromatid exchanges, giving an evidence that it plays an important role in repairing primary DNA damage.

The protective effect of silymarin against photocarcinegenesis was reported by Katiyar *et al* (1997); hepatic damage by Omar *et al* (2007). It lowers glucose and lipid levels in diabetics (LE Magazine 1997&2007). Its clinical properties in the management of hepatic disorders were reported (Wellington *et al*, 2001; and Wen *et al*, 2001)., Its active constituents have been studied (Lee *et al*, 2007).

The drug, at the level of this study, promotes cell proliferation (by enhancing the mitotic activity).

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النشاط المضاد لتكسير المادة الوراثية لعقار سيلمارين

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هدف البحث الحالي لدراسة النشاط المضاد لتكسير المادة الوراثية لعقار السيلمارين. ولتحقيق هذا الغرض تم اختيار الفئران المعملية البيضاء وتم اختيار اربع جرعات من العقار وهي ٢ , ٤ , ٨, ٢٠ ملليجم لكل فئران.
وأجريت الاختبارات التالية:-

1- تقدير التكاثر الخلوي.
2- تحليل الشذوذ الكروموسومي في خلايا نخاع العظام.
3- استحداث تبادل الكروماتيدات الشقيقة في خلايا نخاع العظام.
4- اختبار النواة الصغيرة.
5- تحليل الدور التشتتي في الخلايا الاولية للحيوانات المنوية.

وأظهرت النتائج ان عقار السيمارين يزيد من النشاط الخلوي لدورة الخلية وله نشاط مضاد لتكسير المادة الوراثية كما ظهر ذلك في تحليل الشذوذ الكروموسومي لخلايا نخاع العظام والنواة الصغيرة وامتد أثر العقار للنسيج الجنسي حيث انخفضت معدلات تبادل الكروماتيدات الشقيقة مما يظهر قدرته علي اصلاح الضرر الاولي للمادة الوراثية.