The Possible Protective Role of Lemon Fruit Extract Against Cytogenetic Effects Induced by Cyclophosphamide in Male Albino Mice

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Abstract: The present study aims to evaluate the possible protective effect of lemon fruit extract (LFE) against DNA damage in bone marrow cells was evaluated using micronucleus assay of male mice treated with cyclophosphamide. To attain this aim the 18 male mice be divided into six groups: G1 control group, G2 male mice treated with LFE (10ml/kg /day orally), G3& G4 male mice treated with CP (10 & 20 mg/kg /day intraperitoneally), G5 & G6 male mice dually treated with LFE (10ml/kg/day orally) + CP (10 & 20 mg/kg /day intraperitoneally). All of the abovementioned groups were treated daily for 5 successive days. The micronucleus test showed that CP stimulates the production of micronucleus in polychromatic erythrocytes of bone marrow of treated mice giving evidence that CP is positive clastogen. While dual treatment with LFE and CP showed a reduction in the mean of polychromatic erythrocytes with micronucleus.Therefore, LFE could be concomitantly as a supplement to protect people undergoing chemotherapy.

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1. Introduction

Interest is beginning to mount in assessing the safety of using medications, especially cytotoxic agents that are generally known for its cytotoxic properties that interfere, during the cell cycle, at specific stages of DNA and RNA formation or protein synthesis.

The use of some cytotoxic agents as anti-neoplastic drugs reflects its ability to inhibit proliferation of tumor cells. Its use, however, is associated in most cases with collateral damage of bone marrow and gut mucosal cells(Feig *et al.*, 1994).

Cyclophosphamide, chosen as the subject of this current study, is widely considered as the one of the most important drugs used in the chemotherapy of many malignant tumors, such as Multiple Myeloma, Lymphocytic Leukemia and ovarian adenocarcinoma (Davidson *et al.,* 1990), as well as a number of autoimmune diseases, such as Rheumatoid Arthritis, Hodgkin's disease, Lupus Erythematosis and Scleroderma (Levin and Richie, 1989 and Salem *et al.,* 2012)

Previous studies indicated that cyclophosphamide (CP) requires metabolic activation to exert its cytotoxic impact within the living organism. Such activation is produced by Hepatic Microsomal Cytochrome P450 Oxidase system (Gilman and Rall, 1999 and Wang *et al.,* 2007).

Metabolic activation of cyclophosphamide (CP) results in the production of two cytotoxic metabolites i.e. Acrolein and Phosphoramide Mustard (PMA) (Nau *et al.,* 1982).

It is believed that that Phosphoramide Mustard (PMA) exhibits considerable antineoplastic activity, while Acrolein may be responsible for cyclophosphamide (CP)'s toxic side effects (Honjo *et al.,* 1988 and Kern *et al.,* 2002).

Recent The studies confirm the ability of cyclophosphamide (CP) to generate Reactive Oxygen Species (ROS), which suppress the liver's antioxidant defensive mechanisms (Stankiewicz *et al*., 2002; Bhattacharya *et al*., 2003).

cyclophosphamide (CP) and cyclophosphamide (CP) products of metabolism causing Acute Cystitis and Renal damage has now become common knowledge (Lawson *et al*., 2008). cyclophosphamide (CP) has also been incriminated as the cause of GIT disturbances, such as nausea, vomiting and gastric complications, as well as pronounced hemo-toxicity i.e. Leucopenia and Lupus.

It has also been proven that the use of cyclophosphamide (CP) in the chemotherapy of pediatric tumors can lead to Micro-orchidism and Oligo-spermia at puberty (Aronson, 2006).

The proven genotoxicity and cytotoxicity of cyclophosphamide (CP) has fuelled a drive to find natural products, which possess anti-oxidant bio-components with chemoprotective effects i.e. capability to protect the cells against the toxic impact of cyclophosphamide (CP)'s metabolites e.g. Acrolein and free radicals and can, therefore, be used to protect

against or even prevent the occurrence of side effects, damaging patients' healthy tissues (Ahmadi *et al*., 2008; Hosseinimehr *et al*., 2010; Pratheeshkumar and Kuttan, 2010).

Some studies suggested the use of antioxidant agents in combination with chemotherapeutic agents, because of its ability to limit and contain the cytotoxic damage inflicted on DNA and healthy tissues as a result of chemotherapy (Antunes and Takahashi , 1999), acting as scavengers that trap free radicals, preventing it from reacting with DNA molecules (Ferguson *et al* ., 2004).

Lemons, long known for its rich content of beneficial elements, vitamins and compounds e.g. Phenolic compounds, Hesperidin, Eriocitrin, vitamin E and vitamin C have been proven by previous studies as the repository of considerable antioxidant activity (Minato *et al*., 2003, Atasayara, *et al.*, 2009; Motawi, *et al.*, 2010; Nafees, *et al*., 2015).

Lemons have also been found to possess scavenging capabilities, trapping free radicals (Minato *et al*., 2003).

Vitamins and Flavonoids are also considered as effective complementary therapeutic agents, protecting healthy tissues against the adverse effects exerted by chemotherapeutic agents, without negating its therapeutic efficacy.(Blaylock ,2000).

The objective of this research, therefore, is to study the potential protective effect of lemon fruit extract (LFE) against the mutagenic impact exerted on bone marrow cells as a result of cyclophosphamide (CP) chemotherapy.

2. Materials

Animals

Experiments were conducted on MFI Albino Mice of the species *Mus musculus*, aged 8-9 weeks and weighing 30±3grams, obtained from the animal pound of King Abdul-Aziz University's King Fahd medical center in Jeddah.

The Drug

Cyclophosphamide(CP), commercially known as Endoxan,is a drug used for chemotherapy of cancer patients and is available as powder to be dissolved in a physiological solution and was purchased from Baxter Oncology, Halle,Germany.

Anti-Natural Material

TheLemon fruit (*Citrus limonum* Risso, *Citrus limon* (L.) Burm), (Rutaceae species).

Method

Experimental Desin

Table (1): Shows the number of mice and the dosage/ group

Group I (C)		Group II (L)		Group III (T1)		Group IV (LFE+ T1)	
Number of mice	Dose	Number of mice	Dose	Number of mice	Dose	Number of mice	Dose
3	Physiological solution	3	(10 ml Lemon	3	T1A (CYCLOPHOSPHAMIDE(CP) 10 mg /kg)	3	T2A (L 10 ml / kg + CYCLOPHOSPHAMIDE(CP) 10 mg / kg)
				3	T1B (CYCLOPHOSPHAMIDE(CP) 20 mg /kg)	3	T2B (L 10 ml / kg + CYCLOPHOSPHAMIDE(CP) 20 mg / kg)

C: Control, L:Treatment with Lemon, T1: Treatment with Cyclophosphamide, L+ T1: Treatment with Lemon & Cyclophosphamide

The 18 subject male mice were divided into 6 groups, each containing 3 mice:

The first Group: The control group treated with a physiological solution

The second Group: The group treated with Lemon Fruit Extract at a dose of10ml/kg body weight

The third Group: The group treated with a therapeutic dose 10mg/kg body weight of CP, adjusted into 20ml/kg body weight for mice, in accordance with the international dose conversion table(Paget and Barnes, 1964) (Naghshvar *et al*., 2012).

The fourth Group: The group treated with a combination of LFE + therapeutic dose of CP and LFE+ with double therapeutic dose of CP (Sakr *et al*., 2013)

Treatment

All groups treated with CP had the drug injected intra-peritoneally (Anton, 1997), while LFE was administered by Oral Intubation (O.I.)via an oro-gastric tube (Sakr *et al*., 2013).

Each group was treated daily for 5 consecutive days (Naghshvar *et al*., 2012).

24 hours after the last treatment, subject animals were dissected and their femur bone prepared for genetic and cellular study.

The Micronucleus test

Glass slides were used for this study, where a number of femur bone slices were taken from each of the mice in both control and experimental groups treated with various doses of Physiological solution,

LFE, CP and a combination of both CP and LFE and examined separately.

1000 Polychromatic erythrocytes (PECs) were then taken from each of the prepared samples and the number of micronuclei was counted, observing rules recommended by (Schmid, 1975; Hayashi*et al*., 1984; Albanese and Middleton, 1987) i.e. the radius of each micronucleus must be less than 1/5 the radius of PEC and must as well be round, oval, annular or bean-shaped and must further react with the same dye as the nucleus i.e. be dyed in the same color as the nucleus and must meet the following requirements:

-Micronuclei must acquire a red color, disregarding the associated black dots, that doesn't change with changing microscope's magnifying power

-The cell must have a clear and well-defined borders

-More than 1 micronucleus per cell are counted as one

To calculate the protective (anti-mutagenic) rate of LFE against CP mutagenic effect, as reflected by induction of micronucleus formation the following equation was applied (Serpeloni, *et al*., 2008):

$$100 - \frac{\%\ (MN)\ in\ (CP + LFE)\ groups}{\%\ (MN)\ in\ (CP)\ groups} \times 100$$

Statistical Analysis

Computer software SPSS (version 10), supported on Microsoft windows, was used to enter all data and results in connection with this study and to subsequently conduct statistical analysis, using studen's "t" test to compare results obtained from the groups treated with various doses of CP and from the group treated with a combination of CP and LFE with those obtained from the control group treated with a physiological solution.

ANOVA (Analysis of Variance) was also used to compare the statistical significance of treatment with LFE, treatment with a therapeutic dose of CP, treatment with double CP therapeutic dose and the treatment with a combination of CP and LFE.

Using the least Significant Difference, types of treatment were ranked in terms of the highest effect on the variable.

3. Results

Impact of treatment with LFE

Results obtained 24 hours after the last treatment of male mice with LFE at a dose of 10 ml/kg showed a very slight increase in the number of micronuclei, with no significant differences in the mean appearance of PECs containing micronuclei, compared to the control sample. Results were (6.00±1.15), (0.60%), (4.33± 0.88) and (0.43%) respectively. (Table2, Fig 2)

Impact of treatment with a therapeutic dose 10mg/kg of CP

Results obtained from (Table 2) indicate the high toxic impact exerted by CP on bone marrow cells of male mice treated with a therapeutic dose 10mg/kg of CP . This treatment showed a significant increase ($P \leq 0.001$) in the mean appearance of micronucleus containing PECs, valued at (24.33± 0.88), posting a percentage of 2.43% compared to the control sample.

Impact of treatment with LFE and a therapeutic dose of 10mg/kg CP

Examination of micronucleus containing PECs in bone marrow cells of male mice treated with a combination of LFE at a dose of 10ml/kg and therapeutic dose 10mg/kg body weight of CP indicated a significant decrease($P \leq 0.001$) in the mean appearance of micronucleus containing PECs, valued at (14.00±1.15), posting a percentage of 1.4% compared to (24.33±0.88) and a percentage of 2.43% resulting from treatment with a therapeutic dose 10mg/kg of CP (Table:2).

Upon calculation of the anti-mutagenic impact, based on micronucleus formation or lack thereof, it was found that the combined treatment with LFE and CP had caused 73.79% improvement.

Fig :2 shows the relationship between the impact of individual treatment with either LFE or CP and the treatment with a combination of LFE and CP.

Impact of treatment with DOUBLE the therapeutic dose 20mg/kg of CP

Results obtained from (Table 2) indicate the high toxic impact exerted by CP on bone marrow cells of male mice treated with double the therapeutic dose 20mg/kg of CP. This treatment showed a significant increase ($P \leq 0.001$) in the mean appearance of micronucleus containing PECs , valued at (37.00±3.06), posting a percentage of 3.70% compared to the control sample .

Impact of treatment with LFE and DOUBLE the therapeutic dose 20mg/kg of CP

Results obtained from (Table 2) indicate that the combined treatment with LFE at a dose of 10 ml/kg and double therapeutic dose 20mg/kg of CP showed a significant decrease ($P \leq 0.001$) in the mean appearance of micronucleus containing PECs, valued at (12.00± 1.53), posting a percentage of 1.20%. Upon calculation of the anti-mutagenic impact, based on micronucleus formation or lack thereof, it was found that the combined treatment with LFE and double therapeutic dose of CP had caused 208.33% improvement. Fig: 3 shows the relationship between the impact of individual treatment with either LFE or double therapeutic dose of CP and the treatment with a combination of LFE and CP.

Comparing the impact of treatment with LFE at a dose of 10ml/kg and the combined treatment with LFE at a dose of 10 ml/kg and at a therapeutic dose 10mg/kg bodyweight of CP in terms of ability to induce micronucleus formation, using ANOVA and LSD.

Results obtained from table: 3 indicate a significant difference ($P \leq 0.001$) in the mean appearance of micronucleus containing PECs, valued at (F=79.19) between treatment with LFE at a dose of 10ml/kg, treatment with a therapeutic dose(10mg/kg bodyweight) of CP and the treatment with a combination of LFE and CP as compared with the control sample.

The comparison test, using the least significant difference, showed a significant increase($P \leq 0.001$) in the mean appearance of micronucleus containing PECs as a result of treatment with therapeutic dose of CP, while a highly significant difference of ($P \leq 0.01$) was posted by the combined treatment with LFE and CP. However, treatment with LFE posted no significant difference in the mean appearance of micronucleus containing PECs (Fig: 4). We can, therefore, arrange treatments in terms of highest impact on induction of micronucleus formation as follows:

CP treatment >Combined treatment> LFE treatment

Comparing the impact of treatment with LFE at a dose of 10ml/kg and the impact of the combined treatment with LFE and double therapeutic dose 20mg/kg of CP on the induction of micronucleus formation, using LSD

Results obtained from table: 3 indicate a significant difference ($P \leq 0.001$) in the mean appearance of micronucleus containing PECs , valued at (F=66.55) between treatment with LFE at a dose of 10ml/kg, treatment with double therapeutic dose (20mg/kg bodyweight)of CP and the treatment with a combination of LFE and double therapeutic dose of CP as compared with the control sample.

The comparison test, using the least significant difference LSD, showed a significant increase ($P \leq 0.001$) in the mean appearance of micronucleus containing PECs as a result of treatment with double therapeutic dose of CP, while a highly significant difference of ($P \leq 0.01$) was posted by the combined treatment with LFE and CP. However, treatment with LFE posted no significant difference in the mean appearance of micronucleus containing PECs (Fig : 5).

We can, therefore, arrange treatments in terms of highest impact on induction of micronucleus formation as follows:

CP treatment >Combined treatment> LFE treatment

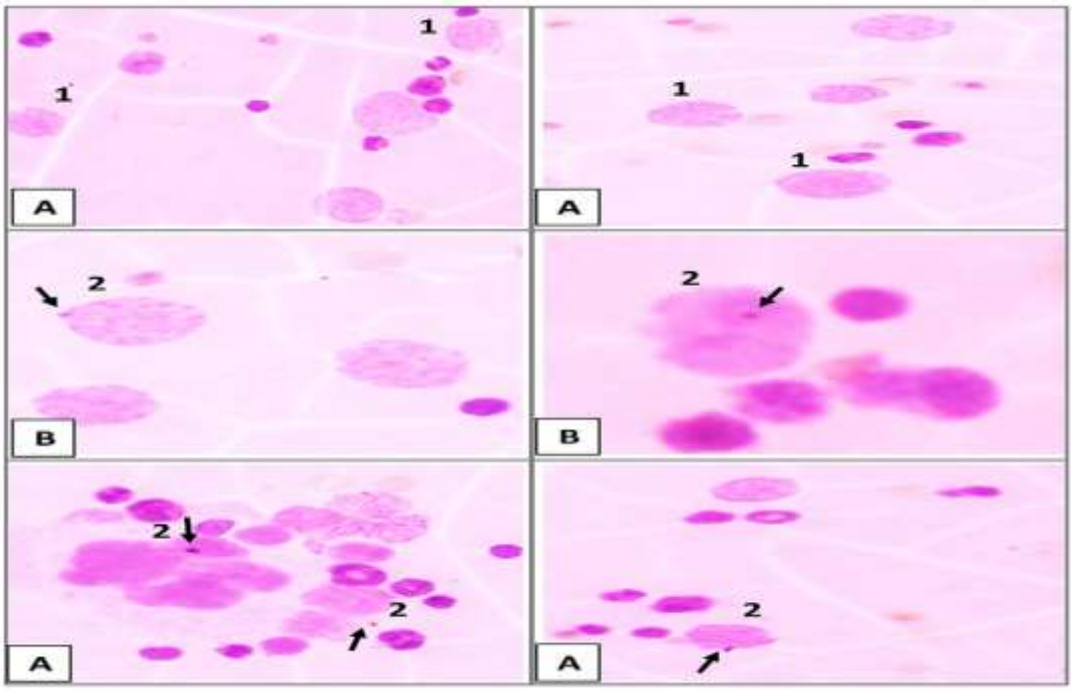

Fig (1) Micrographs show Bone marrow cells in treated male mice and show where

1. Polychromatic erythrocytes "normal"
2. Polychromatic erythrocytes" with micronucleus" A - (X400) B - (X1000)

Table (2): Effect of Lemon, Treatments of (10, 20 mg/kg) of Cyclophosphamide and The Dual Treatment with Lemon and Cyclophosphamide on the mean of polychromatic erythrocytes with micronucleus in Male Mice.

Groups Treatment	No. animal	The calculated number of polychromatic erythrocytes	The number of polychromatic erythrocytes with micronucleus	Mean ± Std.Error	The rate of induction of micronucleus	The rate of antimutagens Effects
C	1 2 3 Mean ± Std.Error	1000 1000 1000 3000	13	4.33± 0.88	0.43%	___
L	1 2 3 Mean ± Std.Error	1000 1000 1000 3000	18	6.00± 1.15	0.60%	___
T1A	1 2 3 Mean ± Std.Error	1000 1000 1000 3000	73	***[a] 24.33±0.88	2.43%	___
T1B	1 2 3 Mean ± Std.Error	1000 1000 1000 3000	111	***[a] 37.00 ± 3.06	3.70%	___
T2A	1 2 3 Mean ± Std.Error	1000 1000 1000 3000	42	***[b] 14.00±1.15	1.40%	73.79
T2B	1 2 3 Mean ± Std.Error	1000 1000 1000 3000	36	*** [c] 12.00 ±1.53	1.20%	208.33

C:Control , L: Lemon(10 ml /kg) ,T1A: Cyclophosphamide(10 mg/kg),T1B : Cyclophosphamide(20 mg/kg),T2A:: Lemon+ Cyclophosphamide (10 ml/kg +10 mg/kg ,T2B: Lemon + Cyclophosphamide (10 ml/kg +20 mg/kg +)

a: Comparison with C , b: Comparison with T1A , c: Comparison with T1B

p* significant<0.05 p** highly significant<0.01 p*** extremly significant<0.001

Table (3) : ANOVA and LSD between The Effect of Treatment of (10,20 mg/kg) of Cyclophosphamide, Lemon and The Dual Treatment with Lemon and Cyclophosphamide on the mean of polychromatic erythrocytes with micronucleus.

	(ANOVA)		(LSD)		
	(F)	(Sig)	Groups Treatment	Mean Difference	(Sig)
Control (C)	79 .19	***	(L)	-1. 67	
			(T1A)	-20.00	***
			(T2A)	-9.67	**
Control (C)	66 .55	***	(L)	-1.67	
			(T1B)	-32.67	***
			(T2B)	-7.67	**

C:Control , L: Lemon(10 ml /kg) ,T1A: Cyclophosphamide(10 mg/kg),T1B : Cyclophosphamide(20 mg/kg),T2A:: Lemon+ Cyclophosphamide (10 ml/kg +10 mg/kg ,T2B: Lemon + Cyclophosphamide (10 ml/kg +20 mg/kg +)

p* significant<0.05 **p**** highly significant<0.01 **p***** extremly significant<0.001

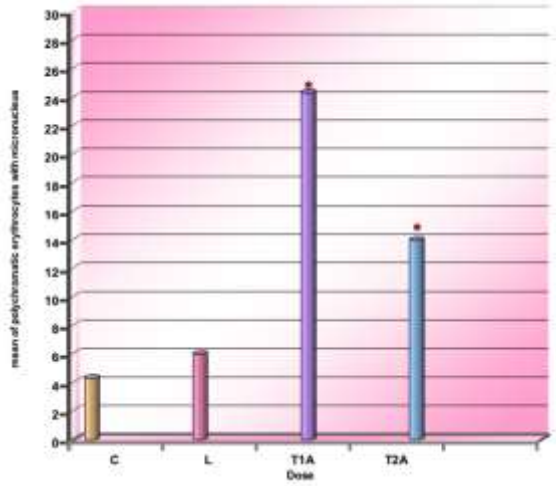

Fig (2): Effect of Lemon, (10mg/kg) of Cyclophosphamide and The Dual Treatment with Lemon and Cyclophosphamide on the mean of polychromatic erythrocytes with micronucleus in Male Mice .

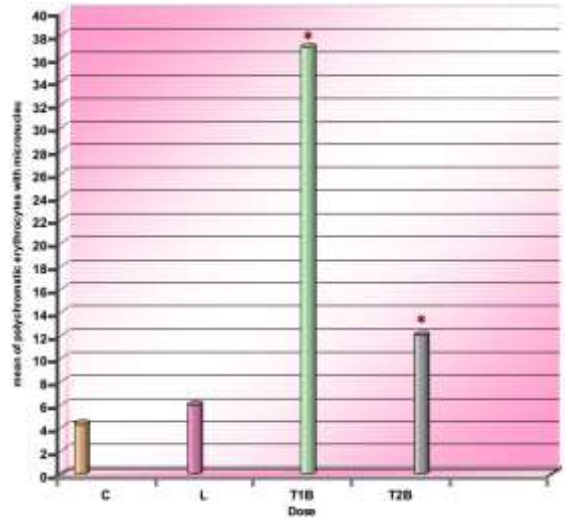

Fig (3): Effect of Lemon, (20 mg/kg) of Cyclophosphamide and The Dual Treatment with Lemon and Cyclophosphamide on the mean of polychromatic erythrocytes with micronucleus in Male Mice

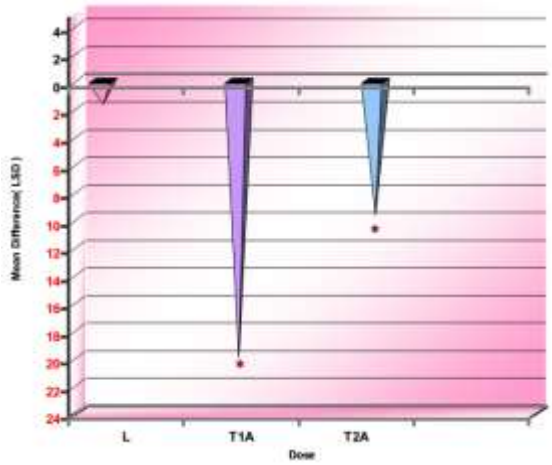

Fig (4): Comparison between The Effect of Treatment of Lemon, (10 mg/kg) of Cyclophosphamide and The Dual Treatment with Lemon and Cyclophosphamide on the mean of polychromatic erythrocytes with micronucleus by LSD.

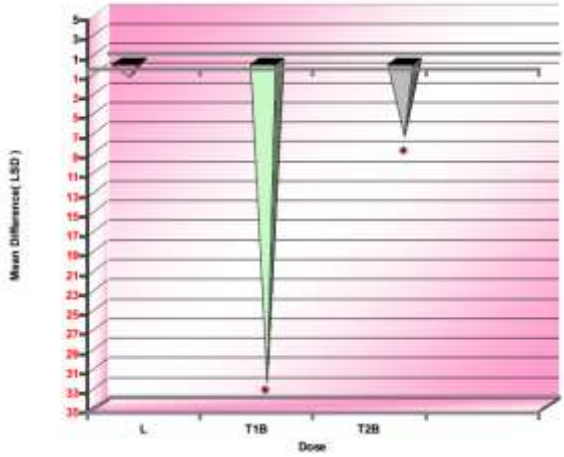

Fig (5) : Comparison between The Effect of Treatment of Lemon, (20 mg/kg) of Cyclophosphamide and The Dual Treatment with Lemon and Cyclophosphamide on the mean of polychromatic erythrocytes with micronucleus by LSD .

4. Discussion

The results of the micronucleus test showed the ability of CP to induce the polychromatic erythrocytes PECs with micronucleus in bone marrow cells of male mice treated with various doses(Figs:2&3)

It also indicated an increase in in PECs with micronucleus formation that is directly proportional to the increase in the dose of CP, which is consistent with findings arrived at by many previous researchers, as a result of bone marrow treatment (Shukla *et al*., 2004).

Previous studies conducted by (Goldberg *et al*, .1983; Albanese, 1987) indicated that upon the intra-peritoneal injection of male mice with various doses of chemotherapeutic agent CP and then taking bone marrow samples at various periods after administration of the dose, the highest response to CP was 24 hours after administration of the dose, as reflected by the highest percentage of PECs with micronucleus.

We have, thereupon, chosen to take bone marrow samples in the current study 24 hours after the administration of the last dose and found a

significant increase in the mean appearance of immature, PECs with micronucleus as compared to the control sample at the time.

Many previous studies demonstrated the ability of numerous chemical compounds to induce PECs with micronucleus in both human and mice. Such chemical compounds include carcinogens (Friedmane and Stub, 1979) and anti-leukemia drugs (Matter and Grauwilet, 1974; Chaueby *et al*., 1978; Yamamoto and Kikuchi, 1981; Richardson and Richold, 1982; Abe *et al*., 1984; Larripa *et al*., 1984; Przybojewska *et al*., 1984)

The Micronucleus test, characterized by being a simple, fast and short term test, in addition to the fact that it is easy to perform and relatively cheap, provides reliable evidence as to the occurrence of genotoxicity.

The appearance of micronuclei in PECs in mice treated with both the therapeutic dose and double therapeutic dose of CP, as borne out by the current study, is therefore credible evidence of the genotoxic and mutagenic impact of CP.

These results are consistent with previous studies conducted by (Chen *et al.,* 1994) and (Edenharder *et al.,* 1998), in which mice were administered CP at a dose of 200mg/kg, resulting in a marked increase in micronucleus count in bone marrow cells.

Shukla *et al.* (2004), Zhang *et al.* (2008),TripathiandJena (2009)conducted experiments on a group of mice injected intra-peritoneally with a 100mg/kg dose of CP, where considerable DNA damage was subsequently observed in bone marrow cells, leading to increased micronucleus count and the development of large numbers of chromosomal aberrations in the bone marrow of subject mice.

Nafees *et al.* (2011) and Rehman *et al.* (2012), as well as Devi and Mazumder(2016) conducted experiments on a group of mice injected intra-peritoneally with a 50mg/kg dose of CP, where considerable DNA strand breakdown as well as much increased micronucleus count was subsequently observed in bone marrow cells 24 hours after CP administration.

Shokrazadeh *et al.* (2013) conducted experiments on a group of mice injected with a 70mg/kg dose of CP, resulting in much increased micronucleus count in bone marrow PECs .

Bhattacharjee *et al.* (2014; 2015) conducted experiments on mice injected with a 25mg/kg dose of CP, resulting in 63.03% increase in ROS, as well as marked increase in DNA splinters in bone marrow cells.

CP belongs to the class of Alkylating agents, which are highly reactive agents, transferring their Alkyl group to the cell's most important parts, by combining with carboxyl, amino, sulfhydryl and phosphate groups (Connors, 1979). Such agents are believed to alkylate DNA at any stage of cell cycle non-specific(CCN), they can be as effective during any stage of the cell cycle, even the quiescent G_0 phase (Lehne *et al*., 1990; Katzung and Trevor, 1995).

Vijayalaxmi and D'souza (2004) also indicated that the most important strategy in cancer therapy depends primarily on the use of Alkylating anti-cancer drugs, which react with DNA molecules, resulting in covalently modified bases.

Alkylating agents, therefore, act either as topoisomerase inhibitors or as free radical generation agents, but in both cases attack DNA molecules, inducing chromosomal aberrations.

The cross links between DNA strands is considered to be the main causative factor in the cytotoxicity of most medically active Alkylating agents, inactivating template DNA strand, thus stopping DNA production and ultimately leading to cell death (Garcia *et al.,* 1988; Erikson *et al.,* 1989).

The ability of Alkylating agents to interfere with the integrity and functionality of DNA molecules in fast dividing tissues demonstrates the basis for both its therapeutic applications as well as its cytotoxic characteristics.

Alkylating agents, while exerting an adverse impact on slow dividing tissues, such as hepatic and renal tissues, exert a an extremely cytotoxic impact on fast dividing tissues, such as bone marrow tissue cells (Padmalatha and Vijayalaxmi, 2001).

There were many studies that highlighted CP reactivity with nucleic acids and proteins, specifically reacting with DNA, creating both intra-strand and inter-strand cross links, with N7 in the Guanine base, being the most CP reactive atom (Surya *et al*., 1978; Erickson *et al*., 1980).Many studies suggested, as well, that Platinum-adenosine -to- guanosine links is the most dangerous linksof all in terms of cytotoxicity (Reed *et al.,* 1986; Parker *et al.,* 1991)These links inhibit both replication and transcription of DNA molecules, resulting in DNA breaks (Nafees *et al.,* 2011) and miscoding(Kishore Reddy *et al.,* 2010).

Alkylation of DNA has thus become a known requirement of the mechanism of action of cytotoxic drugs (Lawley and Philips, 1996).

Broken DNA strands and DNA strand cross links were generally studied in monocytes of the peripheral blood of cancer patients undergoing CP chemotherapy(Hengstler *et al.,* 1992).

Murata *et al.* (2004) explained that the creation of intra-strand and inter-strand cross links between the bases of DNA molecule leads to its damage and eventual destruction and may even result

in apoptosis or programmed cell death(Fritsche *et al.*, 1993)

The fact that alkylating agents are also free radicle generating agents is another important aspect that bears in-depth discussion.

Mazumdar *et al.* (2011) suggested that chemotherapy releases free radicles such as reactive oxygen species, hydrogen peroxide (H2O2), super oxide radical anion and single oxygen radical.

Ray *et al.* (2011) also indicated that CP chemotherapy releases Acrolin radicals, which create DNA cross links, decrease the activity of antioxidant enzymes and oxidize surrounding molecules, such as DNA, lipid and protein molecules, resulting in many morbidities such as aging and cancer (Feig *et al*., 1994).

Combined treatment with both LFE and CP, on the other hand, caused a clear reduction in the numbers of micronuclei in PECs , estimated at 73.79% with CP therapeutic dose (10mg/kg body weight) and **208**.33% with double therapeutic dose of CP (20mg/kg body weight). This result is consistent with the findings of previous studies, which indicated the possibility of preventing the harmful effects exerted by CP on bone marrow cells, by using compounds that possess antioxidant properties and have, therefore, the ability to limit the cytotoxic impact associated with CP chemotherapy.

Alvarez-Gonzales *et al.* (2001) studied the impact of Naringin, a flavonoid element that occurs naturally in grapefruit, on cytotoxicity resulting from treatment with Ifosphamide.

Naringin was administered orally at 50, 250 and 500 mg/kg doses to subject mice, which were injected an hour later with a 60 mg/kg dose of Ifophosphamide. Blood samples were then taken from the tail before and after chemotherapy by 24, 48, 72 and 96 hours, on which to conduct the micronucleus test. The combined treatment exerted a clear inhibitory impact on micronuclei resulting from Ifosphamide treatment.

Jagetia and Reddy (2002) also conducted a study on the protective impact of Naringin,a flavonoid element that occurs naturally in grapefruit, against cytoxicity in bone marrow of miceexposedto gamma radiation. In this study, a marked reduction in micronucleus count was observed in bone marrow cells of subject mice intra-peritoneally injected with Naringin 45 minutes prior to exposure to Gamma radiation.

Hosseinimehr *et al.* (2003), as well, conducted a study on a group of mice injected intraperitoneally with 250, 500 and 1000mg/kg doses of the citrus extract(*Citrus aurantium* var. amara)one hour before exposure to Gama radiation.A marked reduction in post-radiation micronucleus count was observed in bone marrow PECs .

Shokrzadeh *et al.* (2015) mentioned that Hesperidine, a flavonone glycoside, played a role in reducing micronucleus count in human lymphocytes, originally increased as a result of treatment with Diazinon. In this study, blood samples collected from 5 volunteers were incubated with Hesperidine for 3 hours and then 750 mg/kg Diazinon were added and the mixture further incubated for 24 hours.

CP chemotherapy will, therefore, result in impairment of cellular protective mechanism and increase in oxidation processes, Which are the result of releasing free radicles - a known for their destructive cells and then tissue. CP considered as an strong oxidizing agent, in its reducing antioxidants and depleting antioxidant enzymes, particularly Glutathione (GSH), in various tissues. Glutathione is known as its ability to scavenger free radicle trapping and also for its ability to counter cytotoxicity caused by treatment with chemical compounds, including chemotherapeutic agents.

LFE, on the other hand, can be considered as a potent antioxidant, limiting cytotoxic effects resulting from treatment with CP, because of its high content of antioxidants such as vitamins E and C, which induce synthesis and release of Glutathione and Flavonoids e.g.Hesperidine, Eriocitrin and Rutin and Catechins, which help increase the effectiveness of cellular auto-protective mechanism against oxidative destruction, because of its natural contents which act as free radicle scavengers.

Based on the foregoing, we recommend that LFE be administered in combination with anti-cancer chemotherapy to limit the harmful impact of chemotherapeutic agents without interfering with their therapeutic effect.

We further recommend the ingestion of dietary supplements, containing vitamins and flavonoids that act as free radicle scavengers in the body.

References

1. Abe, T., Isemura, T., and Kikuchi, Y. (1984) Micronuclei in Human Bone-Marrow Cells: Evaluation of The Micronucleus Test Using Human Leukemia Patients Treated with Antileukemic Agents. Mutat. Res., 130(2): 113-120.
2. Ahmadi, A., Hosseinimehr, S.J., Naghshvar, F., Hajir, E. and Ghahremani, M. (2008) Chemoprotective Effects of Hesperidin Against Genotoxicity Induced by Cyclophosphamide in Mice Bone Marrow Cells. Archives of Pharmacal Research. 31: 794-797.
3. Albanese, R. (1987) The Cytonucleus Test in The Rat: a Combined Metaphase and Micronucleus Assay. Mutat. Res. 182(6): 309-321.

4. Albanese, R and Middleton, B.J. (1987) The Assessment of Micronucleated Polychromatic Erythrocytes in Rat Bone Marrow. Technical and Statistical considerations. Mutat. Res. 182: 323-332.
5. Alvarez-González, I., Madrigal-Bujaidar, E., Dorado, V., Espinosa-Aguirre, J.J. (2001) Inhibitory Effect of Naringin on The Micronuclei Induced by Ifosfamide in Mouse and Evaluation of Its Modulatory Effect on The Cyp3a Subfamily. Mutation Research. 480-481: 171-178.
6. Anton, E. (1997) Ultrastructural Changes of Stromal Cells of Bone Marrow and Liver after Cyclophosphamide Treatment in Mice. Tissue & Cell. 29(1): 1-9.
7. Antunes, L. and Takahashi,C. (1999) Protection and Induction of Chromosomal Damage by Vitamin C in Human Lymphocyte. Cultures. Teratog. Carcinog. Mutagen. 19(1):53-59.
8. Aronson, J. (2006) Meyler's Side Effects of Drugs: The International Encyclopedia of Adverse Drug Reactions and Interactions, Fifteenth Edition , Oxford, Elsevier, 1025–1032.
9. Atasayara, S., Gurer-Orhan, H., Orhanb, H., Gurelc, B., Girgina, G. and Ozgunes, H. (2009) Preventive Effect of Aminoguanidine Compared to Vitamin E and C on Cisplatin-Induced Nephrotoxicity in Rats. Experimental and Toxicologic Pathology. 61(1); 23–32.
10. Bernrdes, L. (2013) Evaluation of Antimutagenic Effect of Lemon Juice and Acerola on Blood Cells of Mice by Micronucleus Test. Research Supported by FAPESP. 1032: 1-9.
11. Bhattacharjee, A., Basu, A., Ghosh, P., Biswas, J. and Bhattacharya, S. (2014) Protective Effect of Selenium Nanoparticle Against Cyclophosphamide Induced Hepatotoxicity and Genotoxicity in Swiss Albino Mice. Journal of Biomaterials Applications. 29(2): 303-317.
12. Bhattacharjee, A., Basu, A., Biswas, J. and Bhattacharya, S. (2015) Nano-Se Attenuates Cyclophosphamide-Induced Pulmonary Injury Through Modulation of Oxidative Stress and DNA Damage in Swiss Albino Mice. Mol Cell Biochem. 405: 243-256.
13. Bhattacharya, A., Lawrence, R.A., Krishnan, A., Zaman, K., Sun, O. and Fernandes, G. (2003) Effect of Dietary N-3 and N-6 Oils With and Without Food Restriction on Activity of Antioxidant Enzymes and Lipid Peroxidation in Livers of Cyclophosphamide Treated Autoimmune-Prone NZBIW Female Mice. Journal of the American College of Nutrition. 22(5): 388-399.
14. Blaylock, R.L. (2000) A Review of Conventional Cancer Prevention and Treatment and The Adjunctive Use of Nutraceutical Supplements and Antioxidants: Is There a Danger or a Significant Benefit. Journal of American Nutraceutical Association. 3: 75-95.
15. Chaueby, R.C., Kavi, B.R., Chauhan, P.S., and Sandaram, K. (1978) The Effect of Hycanthone and Maleic Hydrazide on The Frequency of Micronuclei in The Bone Marrow Erythrocytes of Mice. Mutat. Res.57(2): 187-191.
16. Chen, S., Xue, K., Ma, G., Wu, J., Wang, H., Xiang, L. and Cheng, N. (1994) Suppressing Effects of Human Fetal Cell Extract on Micronuclei Induced by Cyclophosphamide in mice. Mutation Research. 310: 113-116.
17. Connors, T.A. (1979) The Induction of Cancer by Drug Therapy. In: Drug Toxicology, Gorrod, J.W. (Ed), Taylor and Francisltd, 10-14 Mackin Street, London.
18. Davidson, N., Khanna, S., Kirwan, P. and Naftalin, N. (1990): Long-Term Survival After Chemotherapy With Cisplatinum , Adriamycin and Cyclophosphamide for Carcinoma of The Ovary. Clinical Oncology (Royal College of Radiology). 2: 206-209.
19. Devi, H.P. and Mazumder, P.B. (2016) Methanolic Extract of *Curcuma caesia* Roxb. Prevents The Toxicity Caused by Cyclophosphamide to Bone Marrow Cells, Liver and Kidney of Mice. Phcog Res. 8: 43-9.
20. Edenharder, R., Frangart, J., Hager, M., Hofmann, P. and Rauscher, R. (1998) Protective Effects of Fruits and Vegetables Against *In Vivo* Clastogenicity of Cyclophosphamide or Benzo[a]pyrene in Mice. Food and Chemical Toxicology. 36: 637-645.
21. Erickson, L. C., Ramonas, L. M., Zaharko, D. S. and Kohn, K. W. (1980) Cytotoxicity and DNA Cross-Linking Activity of 4-Sulfidocyclo- phosphamides in Mouse Leukemia Cells *in Vitro*. Cancer Res. 40: 4216-4220.
22. Erickson, J.M., Tweedie, D.J., Ducore, J.M. and Prough, R.A. (1989) Cytotoxicity and DNA Damage Caused by The Azoxy Metabolites of Procarbazine in L1210 Tumor Cells. Cancer Res. 49: 127-33.
23. Feig, D.I., Reid, T.M. and Loeb, L.A. (1994) Reactiveoxygen Pecies in Tumorigenesis. Cancer Res. 54: 1890-1894.
24. Ferguson, L., Philott, M. and Karunasinghe, N. (2004) Dietary Cancer and Prevention Using Antimutagens. Toxicology. 198(1-3): 147-159.
25. Friedman, M.A. and Stub, J. (1979) Induction of Micronuclei in Mouse and Hamster Bone Marrow by Chemical Carcinogens. Mutat. Res. 43(2): 255-261.
26. Fritsche, M., Haessler, C. and Brandner, G. (1993) Induction of Nuclear Accumulation of The Turnersuppressor Protein P53 by DNA Damaging Agents. Oncogene. 8: 307-318.
27. Garcia, S.T., McQuillan, A. and Panasci, L. (1988) Correlation Between The Cytotoxicity of Melphalan and DNA Crosslink as Detected by The Ethidium Bromide Fluorescence Assay in The F1 Variant of B16 Melanoma Cells. Biochem. Pharmacol. 37: 3189-92.
28. Gilman, A.G. and Rall, T.W. (1999) Pharmacokinetics and Side Effects of Cyclophosphamide. Goodman Gillman Pharmacological Basis of Therapeutics. 17: 9–20.
29. Goldberg, M.T., Bihyakey, D.H., and Bruce, W.R. (1983) Comparison of The Effect of 1,2 – Dimethylhydrazine and Cyclophosphamide on Micronucleus Incidence in Bone Marrow and Colon. Mutat. Res. 109: 91-98.
30. Grochow, L.B. and Colvin, M. (1979) Clinical Pharmacokinetics of Cyclophosphamide. Clin. Pharmacokinet. 4: 380-394.
31. Hayashi, M., Sofuni, T. and Ishidate, JR.M. (1984) Kinetics of Micronucleus Formation in Relation to

Chromosomal Abberrations in Mouse Bone Marrow. Mutat. Res. 127: 129-137.
32. Hengstler, J.G., Fuchs, J., and Oesch, F. (1992): DNA Strand Breaks and Dna Cross-Links in Peripheral Mononuclear Blood Cells of Ovarian Cancer Patients During Chemotherapy with Cyclophosphamide/ Carboplatin. Cancer Research., 52: 5622-5626.
33. Honjo, I., Suou, T. and Hirayama, C. (1988) Hepatotoxicity of Cyclophosphamide in Man: Pharmacokinetic Analysis. Res Com Chem Path Pharm. 61: 149-165.
34. Hosseinimehr, S.J., Tavakoli, H., Pourheidari, G., Sobhani, A. and Shafiee, A. (2003) Radioprotective Effects of Citrus Extract Against γ;-Irradiation in Mouse Bone Marrow Cells. J Radiat Res. 44 (3): 237-241.
35. Hosseinimehr, S.J., Ahmadashrafi, S., Naghshvar, F., Ahmadi, A., Ehansalavi, S. and Tanha, M. (2010) Chemoprotective Effects of Zataria Multiflora Against Genotoxicity Induced by Cyclophosphamide in Mice Bone Marrow Cells. Integrative Cancer Therapies. 9: 219-223.
36. Jagetia, G.C. and Reddy, T.K. (2002) The Grapefruit Flavanone Naringin Protects Against The Radiation-Induced Genomic Instability in The Mice Bone Marrow: a Micronucleus Study. Mutation Research. 519: 37–48.
37. Katzung, B.G. and Trevor, A.J. (1995) Cancer Chemotherapy. in: Examination and Board Review Pharmacology. Fourth Edition. Prentice-Hell International Inc. 370-380.
38. Kern, J.C. and Kehrer, J.P. (2002) Acrolein-Indused Cell Death: a Caspaseinfluenced Decision Between Apoptosis and Oncosis / Necrosis. Chem Biol Interact. 139: 79-95.
39. Kishore Reddy, Y.V., Sreenivasula Reddy, P., Shivalingam, M.R. et al. (2010): Testosterone Mediated Partial Recovery of Carboplatin Induced Reproductive Toxicity in Male Wistar Rats. Biomed. Sci. and Res. 2 (1): 46-53.
40. Larripa, I., Mudry De Pargament, M., Labal De Vinuesa, M., Dematti, A. and Brieux De Salum, S. (1984) *In Vivo* and *in Vitro* Cytogenetic Effects of The Anti-Tumor Agent Amsacrina (AMSA). Mutat. Res. 138(1): 87-91.
41. Lawley, P.D. and Phillips, D.H. (1996): DNA Adducts Fromchemotherapeutic Agents. Mutation Res. 355: 13-40.
42. Lawson, M., Vasilaras, A. and De vries, A. (2008) Urological Implications of Cyclophosphamide and Ifosfamide. Scand J Urol Nephrol. 42: 304-317.
43. Lehne, R.A., Crosby, L.J., Hamilton, D.B. and Moore, L.A. (1990): Representative Anticancer Drugs. In: Pharmacology for Nursing Care. Saunders Company-Harcourt Brace Jovanovich, Inc.
44. Levin, A.R. and Richie, J.P. (1989): Urological Complication of Cyclophosphamide. J urology. 141: 1063-1069.
45. Matter, B.E. and Grauwilet, J. (1974) Micronuclei in Mouse Bone Marrow Cells. A Simple *in Vivo* Model for The Evaluation of Drug-Induced Chromosomal Aberrations. Mutat. Res. 23(2): 239-249.
46. Mazumdar, M., Giri, S., Single, S., *et al.* (2011) Antioxidative Potential of Vitamin C Against Chemotherapeutic Agent Mitomycin C Induced Genotoxicity in Somatic and Germ Cells in Mouse Test Model. Biological and Environmental Sciences. 7 (1): 0975-2773.
47. Minato, K., Miyake, Y., Fukumoto, S., Yamamoto, K., Kato, Y., Shimomura, Y. and Osawa, T. (2003) Lemon Flavonoid, Eriocitrin, Suppresses Exercise-Induced Oxidative Damage in Rat Liver. Life Sci. 72(14): 1609-1616.
48. Miyake, Y., Yamamoto, K., Morimitsu, Y. and Osawa, T. (1998) Characteristics of Antioxidative Flavonoid Glycosides in Lemon Fruit. Food Sci Technol Int Tokyo. 4(1): 48-53.
49. Motawi, M.T., Sadik, N.A. and Refaat, A. (2010) Cytoprotective Effects of DL-Alpha-Lipoic Acid or Squalene on Cyclophosphamide-Induced Oxidative Injury: An Experimental Study on Rat Myocarduim, Testicles and Urinary Bladder. Food and Chemical Toxicology. 48(8-9): 2326-2336.
50. Murata, M., Suzuki, T., Midorikawa, K., Oikawa, S. and Kawanishi, S. (2004) Oxidative DNA Damage Induced by a Hydroperoxide Derivative of Cyclophosphamide. Free Radical Biology & Medicine. 37 (6): 793-802.
51. Nafees, S., Ahmad, S., Arjumand, W., Rashid, S., Ali, N. and Sultana, S. (2011) Modulatory Effects of Gentisic Acid Against Genotoxicity and Hepatotoxicity Induced by Cyclophosphamide in Swiss Albino Mice. Journal of Pharmacy and Pharmacology. 64(2): 259-267.
52. Nafees, S., Rashid, S., Ali, N., Hasan, S.K. and Sultana, S. (2015) Rutin Ameliorates Cyclophosphamide Induced Oxidative Stress and Inflammation in Wistar Rats: Role of NFjB/MAPK Pathway. Chemico-Biological Interactions. 231: 98-107.
53. Naghshvar, F., Abianeh, S., Ahmadashrafi, S. and Hosseinimehr, S. (2012) Chemoprotective Effects of Carnosine Against Genotoxicity Induced by Cyclophosphamide in Mice Bone Marrow Cells. Cell Biochem Funct. 30: 569-573.
54. Nau, H., Spielman, H., Lo Turco Mortler, CM., Winckler, K., Ricedel, L. and Obe, G. (1982) Mutagenic, Teratogenic and Pharmacokinetic Properties of Cyclophosphamide and Some of Its Deuterated Derivatives. Mutat Res. 95: 105-118.
55. Padamalatha, R.S. and Vijayalaxmi, K.K. (2001) Tamoxifen acetate induced Sperm Abnormalities in The in *Vivo* Mouse. Genetic Toxocology and Environ Mutagen. 492: 1-6.
56. Paget, G.E. and Barnes, J.M. (1964) Evaluation of Drug Activities and Pharmacokinetics. London:Academic Press. 135-136.
57. Parker, R.J., Gill, I., Tarone, R., Vionnet, J.A., Grunberg, S., Muggia, F.M. and Reed, E. (1991) Platinum- DNA Damage in Leukocyte DNA of Patients Receiving Carboplatin and cisplatin Chemotherapy, measured by Atomic Absorption Spectrometry. Carcinogenesis. 12: 1253-1258.
58. Pratheeshkumar, P. and Kuttan, G. (2010) Ameliorative Action of Vernonia Cinerea L., on Cyclophosphamide-

Induced Immunosuppression and Oxidative Stress in Mice. Inflammo pharmacology. 18: 197-207.
59. Przybojewska, B., Dziubaltowska, E. and Kowalski, Z. (1984) Genotoxic Effect of Ethyl Acrylate and Methyl Acrylate in The Mouse. Evaluated by The Micronucleus Test. Mutat. Res. 135(3): 189-191.
60. Ray, S., Pandit, B., Ray, S.D., Das, S. and Chakraborty, S. (2011) Cyclophosphamide Induced Lipid Peroxidation and Changes in Cholesterol Content: Protective Role of Reduced Glutathione. Iran J Pharm Sci. 7: 255‐67.
61. Reed, E., Yuspa, S.H., Zwelling, L.A., Ozols, R.F. and Poirier, M.C. (1986) Quantitation of Cis-Diammine dichloro platinum II (Cisplatin) -DNA- Intrastrand Adducts in Testicular and Ovarian Cancer Patients Receiving Cisplatin Chemotherapy. J. Clin. Invest. 77: 545-550.
62. Rehman, M.U., Tahir, M., Ali, F., Qamar, W., Lateef, A., Khan, R., Quaiyoom, A., Hamiza, O. and Sultana, S. (2012) Cyclophosphamide-Induced Nephrotoxicity, Genotoxicity and Damage in Kidney Genomic DNA of Swiss Albino Mice: The Protective Effect of Ellagic Acid. Mol Cell Biochem. 365: 119-127.
63. Richardson, J.C., and Richold, M. (1982) An Evaluation of The Mutagenic Potential of 2-(2-4-diaminophenoxy) Ethanol Using The Micronucleus Test. Mutat. Res. 102(4): 357-360.
64. Sakr, S., El-said, M. and El-shafey, S. (2013) Ameliorative Effect of Grapefruit Juice on Amiodarone-Induced Cytogenetic and Testicular Damage in Albino Rats. Asian Pac J Trop Biomed. 3: 573-579.
65. Salem, M., Al-Khami, A., El-Nagaar, S., Zidan, A., Al-Sharkawi, I., Díaz-Montero, M., Cole, D.(2012)Kinetics of Rebounding of Lymphoid and Myeloid Cells in Mouse Peripheral Blood, Spleen and Bone Marrow After Treatment With Cyclophosphamide. Cellular Immunology, 276; 67–74.
66. Schmid, W. (1975) The Micronucleus Test. Mutat. Res. 31: 9-15.
67. Serpeloni, J.M., Bisarro dos Reis, M., J., Campaner dos Santos, L., Vilegas, W., Varanda, E.A., *et al* (2008) *In vivo* Assessment of DNA Damage and Protective Effects of Extracts from *Miconia* Species Using the Comet Assay and Micronucleus Test. Mutagenesis. 23: 501-7.
68. Shokrzadeh, M., Chabra, A., Naghshvar, F. and Ahmadi, A. (2013) The Mitigating Effect of *Citrullus colocynthis* (L.) Fruit Extract Against Genotoxicity Induced by Cyclophosphamide in Mice Bone Marrow Cells. The ScientificWorld Journal. 2013: 1-8.
69. Shokrzadeh, M., Ahmadi, A., Ramezaninejhad, S. and Shadboorestan, A. (2015) Hesperidin, a Citrus Bioflavonoid, Ameliorates Genotoxicity-Induced by Diazinon in Human Blood Lymphocytes. Drug Res (Stuttg). 65(2): 57-60.
70. Shukla, Y., Srivastava, B., Arora, A. and Chauhan, L. (2004) Protective Effects of Indole-3-Carbinol on Cyclophosphamide-Induced Clastogenecity in Mouse Bone Marrow Cells. Human & Experimental Toxicology. 23: 245-250.
71. Stankiewicz, A., Skrzydlewska, E. and Makiela, M. (2002) Effects of Amifostine on Liver Oxidative Stress Cused by Cyclophosphamide Administration to Rats. Drug Metabolism and Drug Interactions. 19(2): 67-82.
72. Surya, Y. A., Rosenfeld, J. M. and Hillcoat, B. L. (1978) Cross-Linking of DNA in L1210 Cells and Nuclei Treated With Cyclophosphamide and Phosphoramide Mustard. Cancer Treat. Rep. 62: 23-29.
73. Tripathi, D.N. and Jena, G.B. (2009) Intervention of Astaxanthin against Cyclophosphamide-Induced Oxidative Stress and DNA Damage: A Study in Mice. Chemico-Biological Interactions. 180: 398-406.
74. Vijayalaxmi, K.K. and D'souza, M.P. (2004) Studies on The Genotoxic Effects of Anticancer Drug Carboplatin in *in Vivo* Mouse. Int. J. Hum. Genet. 4: 249-255.
75. Wang, X., Zhang, J. and Xu, T. (2007) Cyclophosphamide as a Potent Inhibitor of Tumor Thioredoxin Reductase *In Vivo*. Toxical Appl Pharmacol. 218: 88-95.
76. Yamamoto, K.I. and Kikuchi, Y. (1981) Studies on Micronuclei Time Response and on the Effect of Multiple Treatments of Mutagens on Induction of Micronuclei. Mutat. Res. 90(2): 163-173.
77. Zhang, Q.H., Wu, C.F., Duan, L. and Yang, J.Y. (2008) Protective Effects of Total Saponins From Stem and Leaf of *Panax ginseng* Against Cyclophosphamide-Induced Genotoxicity and Apoptosis in Mouse Bone Marrow Cells and Peripheral Lymphocyte Cells. Food and Chemical Toxicology. 46: 293-302.

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