Study of the possible protective effect of lemon fruit extract against the histopathological changes induced in kidney of male mice treated with cyclophosphamide

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Abstract: The current study aims to evaluate the possible protective effect of lemon fruit extract against the histopathological changes induced in kidney of male mice treated with cyclophosphamide. To achieve this goal a total of 30 male mice were divided into six groups: G1 control group, G2 treated with LFE (10ml/kg b wt.), G3 treated with CP (10mg/kg b wt.), and G4 treated with CP (20mg/kg b wt.), G5 treated with LFE (10ml/kg) +CP (10mg/kg), G6 treated with LFE (10ml/kg) + CP (20mg/kg). All of the abovementioned groups were treated daily for five consecutive days. The examination of kidney tissue of the CP treated male mice, demonstrated several histopathological changes of which intensity depended on the increase of the dose. Severe bleeding in interstitial f the kidney with a defect in the normal structure of most nephron was also noticed. Furthermore, confusion in the order of the cells forming the distal convoluted tubules has occurred along with the in ability to view the intercellular membrane and the disappearance of the inner lumen of most of them. While the co-administration treatment of lemon extract with CP should clear response, the kidney restored some of its normal structure, such as the disappearance of signs if inner bleeding, and glomerulus and renal tubules have become semi-normal to some extent.

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1. Introduction

One of the most common problems faced by those working in the chemotherapy is the toxic effects caused by drugs on normal tissues of patients ***(Hung, 2009)***. Since the cyclophosphamide (CP) is one of the important drugs pervasive in the chemotherapy against many tumors such as lymphocytic leukemia, multiple myeloma and ovarian carcinoma ***(Davidson et al., 1990)***. It's also used in the treatment of autoimmunity diseases such as Rheumatoidarthritic, **Hodgkin's** disease, Scleroderma, Lupus erythrometosis ***(Levin and Richie, 1989)***.

However, the studies have shown that it has side effects, as it causes numerous severe cytotoxic to normal cells, whether in human or experimental animals) Fraiser *et al.*, ***1991; Gokhale et al., 2003)*** such as haemorrhagic cystitis, pulmonary fibrosis, gastrointestinal bleeding ***(Gilman and Rall, 1999)***. Previous studies suggested that CP drug, for its effect to appear in vivo, metabolic activation is required by the hepatic microsomal cytochrome P450 oxidase system ***(Gilman, 1999; Wang et al., 2007)***, resulting in the formation of two metabolically cytotoxic products, namely; phosphoramide mustard and Acrolein ***(Nauet al., 1982)***and it is believed that phosphoramide mustard has a counter cancerous tumors growth (antineoplastic) activity, while the acrolein compound is probably accountable for toxic side effects caused by cyclophosphamide ***(Honjo et al., 1988; Kern and Kehrer, 2002)***. Recent studies confirm that the CP drug is capable of generating reactive oxygen species (ROS) that defy and repress the mechanisms of the liver's antioxidant defense ***(Stankiewicz et al., 2002 ; Bhattacharya etal., 2003)***, and it has become now known that cyclophosphamide or its metabolic products cause acute inflammation in the urinary bladder and may cause renal damage ***(Lawson et al., 2008)***, and ***Sugumar et al. (2007)*** identified the renal damage in mice with the following histopathological signs: glomerular inflammation, the appearance of epithelial cytoplasmic vacuolization in cortical tubules, interstitial edema, and mild hemorrhagic changes in the renal cortex with biochemical changes such as the reduced renal glutathione level and an increase in renal melondialdehyde rate after 24 hours of treatment with cyclophosphamide drug. This was confirmed by the studies, as it was noticed by ***Abraham et al. (2007)*** that when female rats were treated with a single dose of 150 mg/ kg of cyclophosphamide, such treatment led to glomerular inflammation, the appearance of epithelial cytoplasmic vacuolization in cortical tubules, interstitial edema resulting in progressive renal damage.

As for the impact of cyclophosphamide on the renal glutathione rate, many studies have shown that

treatment of rats or mice with an acute and single dose of the cyclophosphamide caused a depletion of renal GSH levels and antioxidant enzyme activities, as well as nephrotoxicity***(Abraham and Isaac, 2010; Hamsa and kuttan, 2011; Rehman et al., 2012)***. In view of cellular toxicity caused by cyclophosphamide, it is now necessary the trend to find natural products have within its components vital antioxidant elements, have chemoprotective effects capable to protect cells from the toxic effects of the metabolic outputs of the drug, (such as acrolein and free radicals) and using them during the chemical treatment in order to protect or prevent the occurrence of side effects that damage normal tissue of patients ***(Ahmadi et al., 2008; Pratheeshkumar and Kuttan, 2010; Hosseinimehr et al., 2010)*** and as the lemon fruit is one of the fruits rich of elements, vitamins and vital compounds such as flavonoid, citrate, phenolic compounds, hesperidin, eriocitrin, vitamin E and vitamin C, which the previous studies have proved that these elements and compounds such as eriocitrin, have anti-oxidant activity ***(Miyake et al., 1998)*** and have the ability to scavenge free radicals ***(Minato et al., 2003).Blaylock (2002)*** has demonstrated that the vitamins and flavonoids are considered complementary therapy that can protect normal tissues against the adverse effects of the chemotherapy agents without negating therapeutic efficiency, thus, the goal of the research is to study the possible protective effect of the Lemon fruit extract (LFE) against histopathological changes induced in the kidney of male mice as a result of treatment with the cyclophosphamide.

2. Materials and Methods

Animals used

The experiments of the research were conducted on a group of male albino mice ***(Musmusculus, 2n = 40)*** of MFI strain between 8-9 weeks of age, with weights ranging between 30±3 g, obtained from the animal house of the King Fahd Medical Center in King Abdulaziz University, Jeddah, where the mice were placed in special plastic cages, inside a well-ventilated room, where temperature was about 22ºC±1º C approximately, and humidity ranging between 45%- 75%, with suitable 12 hours lighting during the daytime, and 12 hours darkness at night-time, with water provided daily, and fed with a balanced dry provender special for experimental animals that is provided by the Center.

Materials used

1. Cyclophosphamide (CY) drug

Cyclophosphamide was used in this research, a drug used in chemotherapy for cancer patients, commercially known as (Endoxan®), comes as powder to be dissolved in saline solution or purchased from (Baxter oncology, Halle, Germany).

2. Lemon fruit extract (LFE)

A foodstuff was used in this research of a vegetarian source of well-known health value, that is the lemon fruit (Citrus limonum Risso, Citrus limon (L.) Burm) of the species (Rutaceae) that was obtained from the vegetable shop in the city of Jeddah. The lemon fruit was washed with distilled water and cut into small pieces and put in the fruit juicer (Moulinex type 753) to use the extract when mice treatment.

Methods used

1. Experiment design

Mice were divided into six groups, each group containing five mice

First group G1: The control group, treated with physiological solution

Second group G2: Group treated with lemon fruit extract, (10 ml/kg body weight) **Third group G3:** Group treated with the therapeutic dose of the drug (10 mg/kg body weight).

Fourth group G4: Group treated with double the therapeutic dose of the drug (20 mg/kg body weight) ***(Naghshvar et al., 2012)***

Fifth group G5: Group treated with the therapeutic dose of the drug+ lemon fruit extract

Sixth group G6: Group treated with double the therapeutic dose of the drug+ lemon fruit extract ***(Sakr et al., 2013)***

2. Method of treatment

All groups that have been treated with cyclophosphamide was injected into the peritoneal cavity (intraperitoneal injection) (.IP) ***(Anton, 1997)***, whereas the lemon fruit extract was given by an oral intubation (OI) ***(Sakr et al. 0.2013)*** and all groups were treated daily for five consecutive days ***(Naghshvar et al., 2012)***, after 24 hours of the last treatment, the animals were vivisected, and kidney were taken out to be prepared for histological study.

3. Histological studies

The tissues were fixed in a solution a 10% buffered neutral formalin, embedded in paraffin wax and cut 5mm thickness, and then the slides were stained with haematoxilyn-eosin ***(Mallory, 1900)***, and were examined by an optical microscope Olympus BX51.

3. Results

A. Histological composition of the kidney in (G1) and (G2)

The kidney appears in the cross-section composed of two areas, an external area of dark brown granular appearance (cortex) and an inner area appears radially striped (medulla). The cortex contains spherical objects called Malpighian corpuscles (M.C.)

and many of the convoluted tubules. The Malpighian corpuscle consists of Bowman's capsule (BC) of cub shape, surrounded by two layers (Dual Walled) inter visceral layer and the outer parietal layer lined with simple squamous epithelial cells, and the Malpighian corpuscle is surrounded with a lock of blood capillaries known as Glomerulus (G), and also the cortex contains the Proximal& Distal Convoluted Tubules (PCT)& (DCT). The proximal convoluted tubules are lined with pyramid shaped cells with unclearly defined edges have brush borders and large size, spherical shaped nuclei and dark granular brown pigmented cytoplasm with eosin. The Distal Convoluted Tubules have cavities lined with cubic epithelial cells and with cytoplasm of less density with Eocene dye, Fig. (1a&b).

B. the effect of treatment with cyclophosphamide drug (G3) and (G4)

Theexamination of the cross-sections of the kidney tissue of the male mice treated with cyclophosphamide demonstrated many histopathological changes the intensity of which increased with increasing the dose treated with, when the dose of drug was 10 mg/ kg, it was observed severe bleeding observed in interstitial tissue of the kidney with an imperfection in the natural shape of most units constituting the **Nniferon** and the manifestation of expansion in interstitial tissue, which indicates the presence of oedema. A clear impact has also appeared on the glomeruli in terms of irregularity in its shape in general, and disorder in Bowman's capsule where it became narrow in some places and there is almost adhesion between the membranes lining such capsule, or expansion in other locations, with bleeding in the capillaries composing such glomeruli and the emergence of cellular and bloody seepage in the renal capsule cavity. There has also appeared disorder in many other glomeruli (indicated by the arrow), with the result of a severe atrophy of its size and visceral laceration in their epithelial membranes with severe bleeding inside. In terms of renal tubules, the effect was clear and severe on the Proximal Convoluted Tubules where such tubules have lost its general shape, as confusion occurred in the order of its constituent cells with inability to identify its by-cellular walls and disappearance of the internal cavity for most of them, and the nuclei of these cells appeared abnormal in terms of small size and severity of its pigment (PN) pyknotic, fragmentation (KR) karyorrhexis or decomposition (KL) karyolysis nuclei and their appearance quite pale in color, however there were some cells with natural nuclei. The cells and nuclei of the Distal Convoluted Tubules (DCT) have been severely affected, as is the case in the Proximal Convoluted Tubules (PCT) and there appeared in both many of vacuolizationde composition, and there was loss of the brush edge in some other, as well as haemorrhage- congested vessels and some areas were spotted in the renal tissue have cases of necrosis and gathering of inflammatory cells (Fig. 2a& b).

By increasing the dose to 20 mg/ kg of the drug, severe pathological changes where recorded, as congestion has occurred in the capillaries and blood vessels of the glomeruli (G), and some glomeruli appeared in a state of complete atrophy and irregular shape, and in some cases lost its distinctive round shape, as they are either swollen with almost complete disappearance of its urinal cavity, making it difficult to identify the lining membrane lining such urinary space, or may be very atrophic so that its urinal cavity is roomy, as it was noted the fragmentation of the brush edge of the Proximal Convoluted Tubules (PCT), and in some cases the pyramid shaped epithelial cells lining these tubules have separated from its basement membrane, and lost its borders and its links with neighboring cells and there appeared numerous cytoplasmic vacuolization (vacuolization decomposition), while some cells united with each other forming a dense homogenous mass, while some cells lining the renal tubules appeared fragmented, a case known as tubulorrhexis, besides, pathological changes were observed in the nuclei of these cells, so that the signs of pyknosis have appeared thereon, and the condition may rather increase over to of nuclei fragmentation and disappearance, ***karyorrhexis*** indicating to the destruction and death of cells, and also signs of cellular inflammation emerged by the emergence of many of the lymphocytes around Malpighian corpuscles (M.C) and between the renal tubules, as well as the Distal Convoluted Tubules (DCT) with its cubic epithelial lining cells have appeared in abnormal shape also (Fig. 3a& b).

C. The impact of the dual treatment with lemon and cyclophosphamide (G5) and (G6)

Histological examination of the kidneys of male mice treated with lemon fruit extract with the cyclophosphamide recorded a clear response both in the G5 (Fig. 4a& b) or G6 (Fig5a& b). Kidneys have regained some of its natural manifestations, such as the disappearance of internal bleeding signs, and the renal glomeruli and tubules became almost normal, as most glomeruli (G) shown the restoration of their normal condition in terms of size and regular shape and internal content and disappearance of what have been previously therein as a result of the treatment with the drug alone, of congestion of the blood capillaries and vessels and decomposition that inflicted the epithelial squamous lining, so that the urinary space became free of any urinary infiltrates or bleeding, and it has also been possible the distinction of into Proximal and Distal Convoluted Tubules as the

renal tubules regained its natural shape in both of the cells and nuclei, as well as borders and interdependence existing among these neighboring renal tubules with each other, as well as the disappearance of the severe congestion between the renal tubules, as a result of treatment with the drug alone, and necrosis cases have decreased in many areas of the kidney with the apparent absence of most of the lymph pellets that have emerged around the renal tubules renal due to the severe inflammation previously, with a marked decrease in vacuolization decomposition.

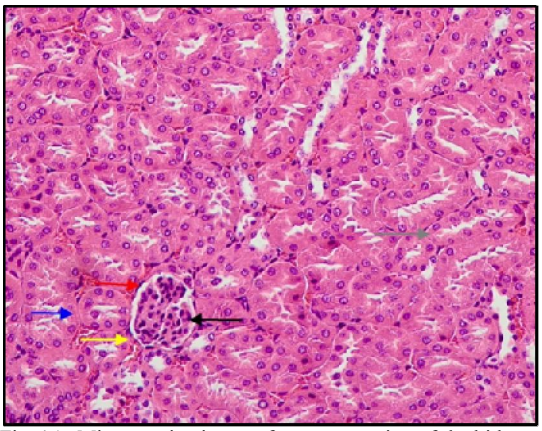

Fig. 1A: Microscopic picture of a cross- section of the kidneys of control group mice (C) of power X200.
Yellow arrow: Malpighian corpuscle.
Red arrow: Bowman's capsule.
Black Arrow: Glomeruli.
Blue arrow:Proximal Convoluted Tubules (P.C.T).
Gray arrow: Distal Convoluted Tubules (D.C.T).

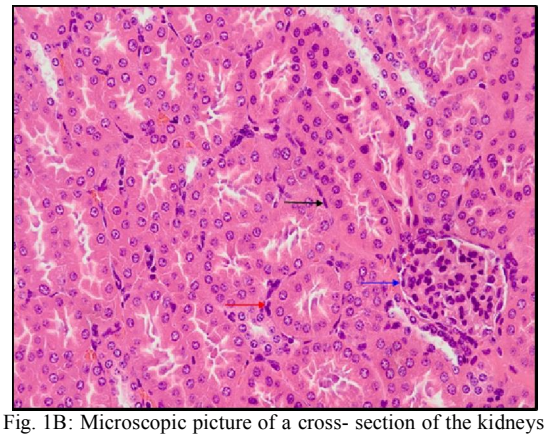

Fig. 1B: Microscopic picture of a cross- section of the kidneys of the group mice treated with (10 ml/kg) of lemon fruit extract, of power X400.
Yellow arrow:Malpighian corpuscle.
Red arrow:Bowman's capsule.
Black Arrow: Glomeruli.
Blue arrow:Proximal Convoluted Tubules (P.C.T).
Gray arrow:Distal Convoluted Tubules (D.C.T).

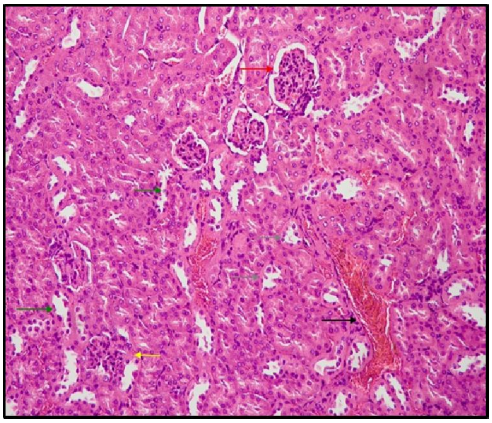

Fig. 2A: Microscopic picture of a cross- section of the kidneys of the group mice treated with (10 mg/kg) of Cyclophosphamide (CP), of power X200.
Red arrow: Expansion in Bowman's capsule cavity.
Yellow arrow: Decomposition in Bowman's capsule.
Black Arrow: Blood congestion in renal tubules.
Grey arrow: Decomposition in proximal convoluted tubules (P.C.T).
Green arrow: Decomposition in distal convoluted tubules (D.C.T).

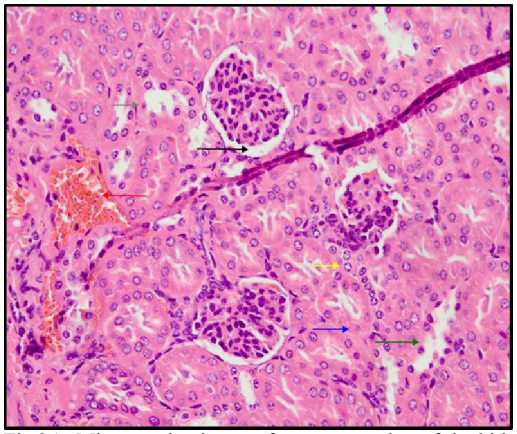

Fig.2A: Microscopic picture of a cross- section of the kidneys of the group mice treated with (10 mg/kg) dose of Cyclophosphamide (CP), of power X400.
Black Arrow: Expansion in Bowman's capsule cavity.
Green Arrow: Decomposition in distal convoluted tubules (D.C.T).
Red arrow:Blood congestion in renal tubules.
Gray arrow: Pyknosis in renal tubules cell nuclei.
Yellow arrow: Karyorrehis in renal tubules cell nuclei

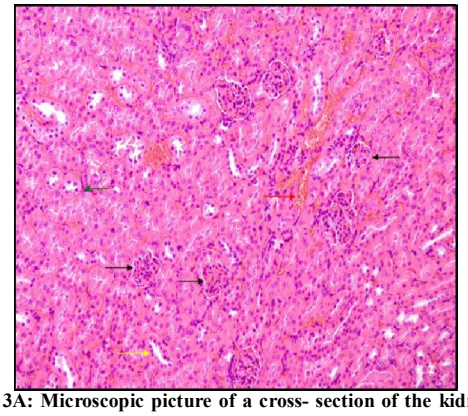

Fig. 3A: Microscopic picture of a cross- section of the kidneys of the group mice treated with a (20 mg/kg) dose of Cyclophosphamide (CP), power X200.
Black Arrow: Malpighian corpuscle.
Green Arrow: Proximal Convoluted Tubules (P.C.T).
Yellow arrow: Distal Convoluted Tubules (D.C.T).
Red arrow: Blood congestion in renal tubules.

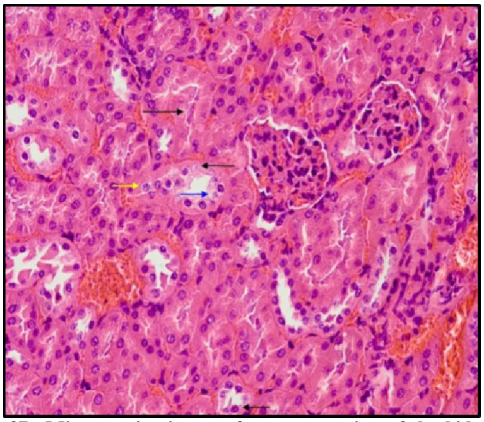

Fig. 3B: Microscopic picture of a cross- section of the kidneys of the group mice treated with a (20 mg/kg) dose of Cyclophosphamide (CP), of power X400.
Yellow arrow: Karyorrehis in renal tubules cell nuclei.
Blue arrow: Karyolysis in renal tubules cell nuclei.
Black Arrow: Death of renal tubules cells.

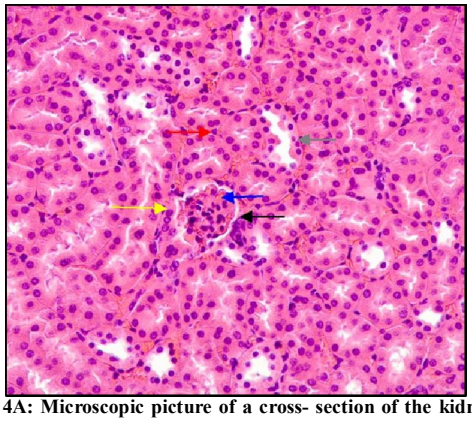

Fig. 4A: Microscopic picture of a cross- section of the kidneys of the group mice treated with a (10 mg/kg) dose of Cyclophosphamide (CP), with (10 ml/kg) of lemon fruit extract of power X200.
Yellow Arrow: Malpighian corpuscle.
Black Arrow: Bowman's capsule.
Blue Arrow: Glomeruli.
Red Arrow: Proximal Convoluted Tubules (P.C.T).
Grey Arrow: Distal Convoluted Tubules (D.C.T).

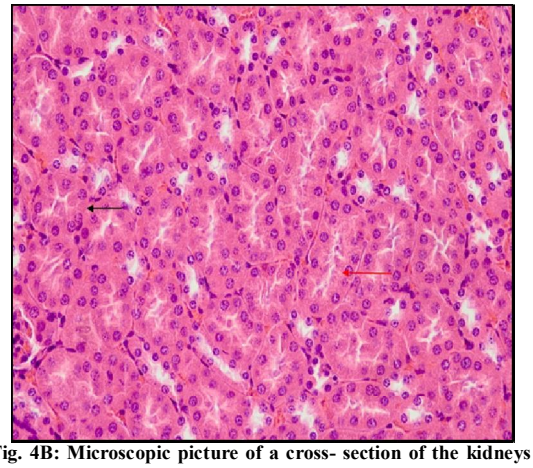

Fig. 4B: Microscopic picture of a cross- section of the kidneys of the group mice treated with a (10 mg/kg) dose of Cyclophosphamide (CP), with (10 ml/kg) of lemon fruit extract of power X400.
Black Arrow: Proximal Convoluted Tubules (P.C.T).
Red Arrow: Distal Convoluted Tubules (D.C.T).

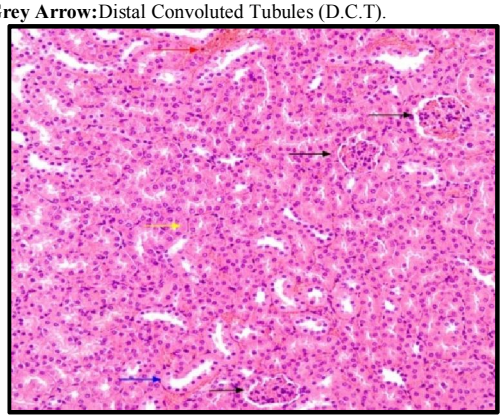

Fig. 5A: Microscopic picture of a cross- section of the kidneys of the group mice treated with a (20 mg/kg) dose of Cyclophosphamide (CP), with (10 ml/kg) of lemon fruit extract of power X200.
Black Arrow: Malpighian corpuscle.
Yellow arrow: Proximal Convoluted Tubules (P.C.T).
Blue arrow: Distal Convoluted Tubules (D.C.T).
Red Arrow: Blood congestion in renal tubules.

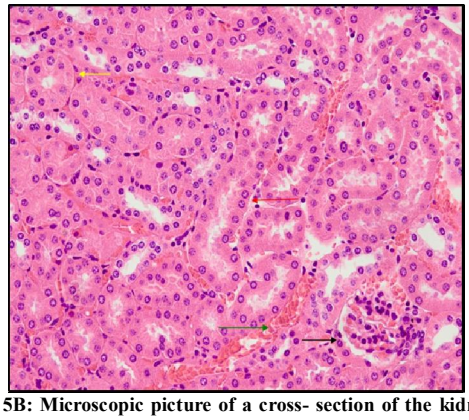

Fig. 5B: Microscopic picture of a cross- section of the kidneys of the group mice treated with a (20 mg/kg) dose of Cyclophosphamide (CP), with (10 ml/kg) of lemon fruit extract of power X400.
Black Arrow: Malpighian corpuscle.
Yellow arrow: Proximal Convoluted Tubules (P.C.T).
Red Arrow: Distal Convoluted Tubules (D.C.T).
Green Arrow: Blood congestion in renal tubules.

4. Discussion

The findings that have been reached in this research showed that the cyclophosphamide has caused histopathological changes in the kidneys of the male mice treated with different doses of the drug compared to the control group, and the severity of these changes were directly proportional to the dose given. Where histological examination of the kidneys of male mice treated with the cyclophosphamide showed an imbalance in their composition, so that most Malpighian corpuscles (M.C) have emerged as abnormal in size and composition, thus glomeruli were severely oedema causing disappearance of the membranes lining such pellets, or very atrophic leading to widening of the urinary space, as well as Bowman Capsules appeared irregularly shaped, and it was noted the presence of infiltrates and hemorrhage and congestion of the blood vessels circulating in theinterstitial tissue with occurrence of decomposition and metamorphoses in the epithelial cells lining the renal proximal and distal tubules, and the emergence of necrosis areas having numerous inflammatory cells, and the emergence of vacuolization decomposition in the form of cytoplasmic vacuolization in many of the epithelial cells lining the renal tubules, with the contraction of their nuclei, pyknosis nuclei. ***Ariens et al. (1976)*** explained that the tissue toxicity appears in the tissue sectors in the form of cellular decomposition accompanied by the composition of cytoplasmic vacuolization and fatty gatherings and tissue necrosis. ***Mollendroff (1973)*** explained the formation cytoplasmic vacuolization within the tissue as cellular defense mechanism against harmful substances crossing the cells, or particles that have been broken, as it collect them in order to prevent them from interfering with the metabolic cell activity, and the appearance of cytoplasmic vacuolization in cells, representing the beginnings of autolysis.

Perhaps what showed up in the results of our study confirm what ***Sugumar et al. (2007)*** has identified, that the kidney damage has histopathological indications, namely: glomeruli inflammation and the emergence of epithelial cytoplasmic vacuolization in cortical tubules, interstitial edema and mild hemorrhagic changes in the renal cortex. Histopathological changes observed in the study are consistent with what previous studies of cyclophosphamide the drug have made reference to, where ***Abraham et al. (2007)*** had noticed that the treatment of female mice with a single sharp dose of 150 mg/ kg of CP drug led to glomeruli inflammation and the emergence of epithelial cytoplasmic vacuolization in cortical tubules, interstitial edema, resulting in progressive damage in the renal tissue. ***Hamsa and kuttan (2011)*** also have indicated to that when treating mice with a sharp dose of 150 mg/ kg of CP drug, such treatment caused glomeruli swelling and irregularity in the composition of Bowman Capsules, and the hydrolysis in the cells lining the cortical tubules and the emergence of necrosis areas in interstitial tissue with a gathering of inflammatory cells.

This is reported also by ***Rehman et al. (2012)*** in their study on male mice when treated with a single 50 mg/ kg dose of CP, the treatment has created renal toxicity with manifestations of large and diffuse decomposition in the renal tubules and also congestion in these tubes, swelling and necrosis, congestion in the glomeruli and an invasion of inflammatory cells to the area of renal cortex. Since many of the studies have indicated that of the important reasons and leading to the emergence of cases of cellular toxicity is the effect on glutathione levels in different organs of the organism, especially the liver, kidney and serum. This is what ***Micheli et al. (1992)*** had confirmed in their study conducted on mice, in which the explained that the glutathione depletion (GSH) to about 20% of its total amount, hinders the cell's ability to defend itself against the toxic effects of the drugs, which eventually may lead to cell destruction and death due to the importance of the glutathione and the enzyme system related thereto, in the processes of removing cellular toxicity ***(Bompart, 1990)***.

Since the cyclophosphamide, which is under study, has proven through previous research, able to decrease glutathione levels in the kidneys of mice and its impact on the activities of antioxidant enzymes in the kidney, such as glutathione-s-tranferase (GST), glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT) ***(Hamsa and Kuttan, 2011; Sinanoglu et al., 2012)***, leading to the emergence of renal toxicity ***(Rehman et al., 2012)*** given the important role played by glutathione and its enzymatic system with the antioxidant defense system of importance in defensive mechanism against oxidizing agents ***(Miyake et al., 1998; Battacharjee et al., 2014),*** so, it may be a logical explanation for these results that the low rate of glutathione is probably one of the reasons that have caused the damage to the renal tissue. Perhaps the other reason is that the cyclophosphamide drug is one of the strong oxidizing agents working to produce high and significant rate of reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide (H2O2) ***(Patel, 1987; Stankiewicz et al., 2002; Bhattacharya et al., 2003)***.

which many studies have proved its ability to attack cell membranes, destroying them, as well as

discouraging the antioxidant defensive mechanisms ***(Cutler, 1992; Miyake et al., 1998)***, and also many researchers have decided that the cyclophosphamide is a strong alkylating agent ***(Khan et al., 2014; Bhattacharjee et al., 2014)***, and it is believed that one of its metabolic such as acrolein can work on alkalization of the basal sites in DNA, RNA molecule, as well as protein (such as: OH^2 - SH - NH2 and COOH) thus leading to the emergence of cellular and genetically toxic effects ***(Ponticelli and Passrrini, 1991)***.

In regards of the dual treatment with lemon fruit extract and cyclophosphamide drug, the histological examination of the kidneys of these mice showed over a clear improvement as the renal tissue has regained, for a large extent, the methodical composition, so that, most Malpighian corpuscles (M.C) seemed natural in shape and composition, infiltration and bleeding disappeared, and severe congestion in the renal tubules and cases of necrosis of such tubules cells have clearly decreased. Such a protective effect many researchers observed when they use different compounds against the renal toxic effect caused by CP, and a few examples on that include Ellagic acid ***(Rehman et al., 2012)***, Spirulina substance, ***(Sinanoglu et al., 2012)***, medical plant *Ipomoea*obscura***(Hamsa and Kuttan, 2011)***, as well as the natural compound Glutamine ***(Abraham and Isaac, 2010)***.

Perhaps the improvement appeared in the renal tissue in the current study as a result of using the lemon fruit extract with cyclophosphamide can be explained by the fact that the lemon fruit is of the fruits rich with vital contents of elements and compounds such as flavonoids and phenolic compounds, hesperidin, eriocitrin and vitamin C and E, which previous research have proved that they all possess anti-oxidant activity, and work to increase the concentration of antioxidant enzymes ***(Miyake et al., 1998; Minato et al., 2003)*** and have important physiological functions as anti-inflammatory and anti-carcinogenic ***(Bracke et al., 1994)***. Studies have also demonstrated the importance of vitamin C as an anti-clastogenic and anti-mutagenic ***(Anderson et al., 1995)*** and strong antioxidant ***(Rao, 1997)***, where all those elements and bio compounds as scavengers for free radicals preventing them from destroying cells and tissues.

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