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Study of Physiological, Histological and Immunological Changes in Female Rats Exposed to Bisphenol-A- and Protective Role of Grape Skin

A Thesis

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By

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بسم الله الرحمن الرحيم

يُنبِتُ لَكُم بِهِ الزَّرْعَ وَالزَّيْتُونَ وَالنَّخِيلَ وَالْأَعْنب وَمِن كُلِّ الثَّمَرت إِنَّ فِي ذَٰلِكَ لَآيَةً لِّقَوْمٍ يَتَفَكَّرُونَ

صدق الله العلي العظيم

سورة النحل

ايه 11

DEDICATION

Dedicated To

My Dear Father

My Dear Mother

My Dear Brothers

My Dear Sister

QASIM

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First and foremost, I wish to express my sincere gratefulness and deep cordial thanks to ALLAH, The Originator of Heavens and Earth, Who provides me the strength and the patience to complete this work.

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Qasim

Summary

The present study was conducted at College of Science in cooperation with Veterinary Medicine College /University of Karbala to investigate the effects of exposing to Bisphenol A (BPA) on some hematological, biochemical, immunological, hormonal parameters in addition to histological changes in different organs of albino female rats.

The experiment extends for 30 days mature female rats (14-16weeks old) were divided (40 female rats) into five groups (eight for each group)(two control groups and three treatment groups). The first group served general control (GC) group and gavaged animals orally with 0.5 ml/Kg /BW of normal saline daily, the second group gavaged orally in corn oil suspension only with 0.5ml/kg BW and served vehicle control (VC) group, the third group served as a treatment1 (T1) their diet mixed with 5% grape skin powder and not gavage, the forth group (treatment 2 group) (T2) their rats orally drenched with 250 mg/kg/BW of BPA suspended in corn oil daily, the fifth group served as a treatment3 (T3) their rats orally drenched with 250 mg/kg/BW of BPA suspended in corn oil daily and their diet mixed with 5% grape skin powder.

The results of present study showed that treatment (2) group caused significant ($p \leq 0.05$) decrease in Hb concentration and RBCs count but the WBC count and neutrophils % were significantly ($p \leq 0.05$) increased in female rats treated with BPA compared with all other groups, while the animals of treatment (3) group appeared significant enhancement in all hematological parameters represented in significant raise ($p \leq 0.05$) in RBCs count and Hb level in addition, significant decrease ($p \leq 0.05$) in WBCs count and neutrophils % in contrast with BPA alone ,the means were reach levels close to that recorded in control and treatment (1) groups in some hematological parameters.

Summary

Results of the biochemical parameters showed that there were significant increase ($p \leq 0.05$) in ALT ,AST levels and level of serum glucose in female rats treated with BPA only, which caused harmful effects on liver and kidney, while treatment (3) group demonstrated that there were significant enhancement ($p \leq 0.05$) in mean of ALT, AST and glucose level compared with group of BPA only but, they were significantly differ($p \leq 0.05$) from that in control groups except glucose level was close to that level of control and treatment (1)groups. Results of immunological indices showed that BPA leads to significant ($p \leq 0.05$) increase in concentrations of C_3, IgG, IL-1β, IL-6, IL-2 and TNF-α compared with all other groups, while grape skin with BPA caused reduced in serum levels of all immunological parameters compared with BPA group alone but they don't reach close to their levels in control and treatment (1) groups. Results of hormones appeared that there were significant increase ($p \leq 0.05$) in estradiol, LH and FSH levels in treatment (2) group, while levels of these hormones significantly ($P \leq 0.05$) lowered in treatment group compared with BPA group alone but they don't reach close to their levels in control and treatment (1) groups. Results of microscopic examination for tissue sections showed presence of histological changes in organs of liver, kidney ,ovary ,uterus and spleen in treatment (2) group represented in presence of pyknotic nuclei in hepatocytes ,massive area of necrosis in the tubular epithelial cells in kidney , presence of cyst-like structures in ovary , increase in uterine luminal epithelial height in uterus and massive necrosis of white pulp in spleen, while animals of treatment (3) group appeared ameliorate in histological changes in studied organs compared with group of BPA only. From the results of the present study, it was concluded that Bisphenol A leads to a negative results on hematological, biochemical, immunological, hormonal parameters in addition to histopathological changes

in organs of liver, kidney, ovary, uterus and spleen, grape skin has protective role in some states to enhancement the harmful effects of BPA e.g. it causes significant decrease in concentrations of C3, IgG, IL-1β, IL-2, IL-6 and TNF-α,grape skin only induce no change in hematological, biochemical, immunological, hormonal parameters in addition to histopathological changes in organs of liver, kidney, ovary, uterus and spleen and Corn oil as a vehicle does not have significant effects in studied parameters compared with general control group.

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List of Abbreviation

Abbreviation term	Meaning
Ab	Antibody
ABC	Avidin-Biotin-Peroxidase Complex
Ag	Antigen
ALT	Alanine Aminotransferase
ANOVA	Analysis of variance
AR	Androgen receptor
AST	Aspartat Aminotransferase
BPA	Bisphenol – A-
BW	Body weight
C3	Complement3
CYP19a	Cytochrome P450 19a
DES	Diethylstilbestrol
DHEA	Dehydroepiandrosterone
E	Eosin
E2	Estradiol
EDCs	Endocrine disrupting chemicals
EDs	Endocrine disrupters
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen receptors
ER-α	Estrogen receptors- alpha
ER-β	Estrogen receptors- Beta
EU	European union
FSH	Follicle stimulating hormone
FPP	Farnesyl-pyrophosphate

GC	**General control group**
GF	**Graafiane follicles**
GGPP	**Geranylgeranyl-pyrophosphate**
GnRH	**Gonadotrophin –releasing hormone**
GOD	**Glucose oxidase**
Hb	**Hemoglobin**
H	**Hematoxyline**
HPGA	**hypothalamic-pituitary-gonadal axis**
HRP	**Horse Radish Peroxidase**
IFN-γ	**Interferon–Gamma**
IgG	**Immunoglobuline G**
IL-1β	**Interleukin-1β**
IL-2	**Interleukin-2**
IL-6	**Interleukin**
KCs	**Kupffer cells**
LD50	**Lethal dose 50**
LH	**Luteinizing hormone**
LOAEL	**Lowest observed adverse effect level**
LSD	**Least Significant Different**
LPS	**Lipopolysaccharide**
MCF-7	**Michigan Cancer Foundation-7**
MCP-1	**Monocyte chemoattractant protein-1**
NIOSH	**National Institute for Occupational Safety and Health**
NO	**Nitric oxide**
OD	**Optical density**
PBS	**Phosphate Buffer Saline**
PF	**Primary follicles**
POD	**Peroxidase**
PVC	**polyvinyl chloride**
RBC	**Red Blood Cells**
RID	**Radial immunodiffusion**
ROS	**Reactive Oxygen Species**
Rpm	**Round per minute**
RSV	**Resveratrol**
SD	**Significant Difference**
SE	**Standard Error**
SF	**Secondary follicles**
SPSS	**Statistical Program for Social Sciences**

TNF-α	**Tumor necrotic factor-α**
TH	**Thyroid hormone**
Th1	**T helper 1**
Th2	**T helper 2**
TR	**Thyroid hormone receptor**
T1	**Treatment 1 group**
T2	**Treatment 2 group**
T3	**Treatment 3 group**
T3	**Triiodothyronine**
VC	**Vehicle control group**
UG	**Uterine glands**
USA	**United States of America**
WBC	**White Blood Cells**

CHAPTER ONE

INTRODUCTION

1-Introduction

Bisphenol-A(BPA) is one of the manufacturing compounds, that introduced in production of different plastic compounds, polycarbonate, become universally used in the production of paper, food and beverage containers, consumer goods and in many other industrial applications (Brotons *et al*., 1995). Recently researches showed that BPA has ability to leach out of some products, include tableware, plastic lining of cans used for food, white dental fillings, sealants and babies' bottles. The leaching was occurred by exposing the plastic to high temperatures (Le *et al.*, 2008).

Kloas (1999) reported that BPA at low doses has action similar to some female hormone (e.g.17b-estradiol, estrone or estriol), and BPA can causes biological effects. As a result, BPA belong to class of chemicals called "hormone disruptors" or "endocrine disruptors",that have ability to disturb the chemical messenger system in the body. Brotons *et al*., (1995) found BPA in the fluid portion of many classes of vegetables such as green beans, mushrooms, mixed vegetables, peas, corn and artichokes, which take from cans with epoxy resin linings.

Tyl *et al*.,*(*2002) and Tyl *et al*.,(2008) demonstrated that BPA at high doses may be lead to changes in liver weight of mice or rats and they showed decreased the viability of rat hepatocytes. Wu *et al., (*2010) reported that Kupffer cells (KCs), which are hepatic macrophages found in the lumen of the liver sinusoids, KCs when activated they released different cytokines and have essential role in the pathogenesis of different liver diseases.

Kopf *et al*., (2010) found that KCs considered as the cause of the inflammatory response, because they lead to release proinflammatory cytokines, include IL-6 and interleukin (IL)-1beta when activated. Yongvanit

et al., (2012) reported that proinflammatory cytokines at high concentrations lead to disruption the homeostasis of oxidants/anti-oxidants and DNA repair enzymes, these proinflammatory cytokines increased in BPA-associated inflammatory processes.

There are several reports indicating that perinatal exposure to BPA in doses below lowest observed adverse effect level (LOAEL) affects reproductive health in adulthood (Durando *et al.*, 2007 and Monje *et al*., 2009). Kamimura *et al.,* (2003) reported that BPA has effect on the immune system of experimental animals, while absence of BPA lead to increase a protective response against infections. AL–Farhaan (2015) reported that there are significant increase in ALT in rats exposed to BPA at high dose because of increase of oxidative stress and damage of liver cells.

On the other hand, grape (*Vitis* spp.) is economically important because its diverse uses in production of grape juice and other food product, the importance of grape products related to their metabolic compositions (Ali *et al*., 2010).

Celotti *et al*., (1996) and Sato *et al.,* (1997) reported that resveratrol is a polyphenolic compound that is found in grape juice and fresh grapes. Resveratrol is localized in the grape skin and considered one of the main components in grape skin; its concentration varies with viticultural practices, specific grape cultivar and climate. Sato *et al.,* (1997) noted that resveratrol at higher concentrations are found in dark-skinned grapes than in light-skinned types. Ellagic acid and myricetin are the major components in the grape skin, However, polyphenols including simple phenols, anthocyanins, stilbenes, proanthocyanidins , flavonoids and vitamin E are the most important class of biologically active compounds in grapes (Xia *et al.,* 2010 and Alzand and

Mohamed,2012).Vattem and Shetty (2005) reported that ellagic acid has protective role because of its antimutagenic, hepatoprotective, high antioxidant and anticarcinogenic qualities.

Vogt (2010) and Ananga *et al.,* (2013) showed that Flavonoids are class of natural polyphenols released by the phenylpropanoid pathway. Harborne and Williams (1992) reported that Flavonoids has defensive role for tissue at oxidative damage or pathogen attack and they play important role in confer UV-protection. In grapes, about 60%–70% of total polyphenols are stored in grape seeds while flavonoids are mainly found in the epidermal layer of grape skin (Hosseinzadeh, 2009; Ali *et al*., 2010 and Tsao, 2010). Conde *et al*., (2007) showed that Flavonoids are the major classes of soluble phenolics in grapes in addition to major contributors of the biological activities in products resulting from grapes. Murphy *et al*., (2011) found that Flavonoids have anti-inflammatory, antimicrobial, antioxidant, cardioprotective and anti-cancer properties.Oral administration of proanthocyanidin (100mg\kg and 200mg\kg) causes non-significant change in thyroid hormones (TH) compared with (-ve) control group but a significant increase compared with (+ve)group. This result was due to improvement of thyroid function. The euthyroid status after administration of proanthocyanidin is due to antioxidant and scavenger effect of free radical (AL-Razag, 2015).

In spite of the development in BPA studies in the last period, the biological and physiological effects of BPA still controversial and required more search. On the other hand, there is a shortage in studies about effects of BPA in Iraq.

Aims of the study

The current study was planned to estimate harmful effects of the exposure to BPA and the possible protective effect of red grape skin against harmful effect of BPA in female rats by study the following parameters:

1. Toxic effect of BPA on some blood and biochemical parameters and probable protective role of grape skin.
2. Investigate the effect of BPA and grape skin on some immunological parameters.
3. Study of histological changes caused by BPA and the ameliorative effect of grape skin.
4. Evaluate effect of BPA on female reproductive system (hormones and organs) and probable improvement of grape skin.

CHAPTER TWO

REVIEW OF LITERATURE

2-Literature review

2-1-Bisphenol-A-

Bisphenol-A- (BPA) is one of the most important industrial chemical introduced basically in polycarbonate plastics and in the epoxy resins, it also widely used in manufacture food and drink cans (Brotons *et al*., 1995). Exposure to BPA as result of hydrolysis of the ester bonds that are link BPA molecules in polymer this hydrolysis of the ester bonds accelerated when temperature increases and in acidic or basic environment. BPA leeches from polycarbonate products significantly as a consequence to repeat washing, exposure metal and plastic cans to heat and/or acidic or basic conditions (Krishnan *et al.*, 1993 and Kang *et al.*, 2003).

2-1-1- History of bisphenol A (BPA)

In 1891, BPA was synthesized firstly but reported synthesis was in 1905. It was firstly reported that BPA has estrogenic features in the 1930's. However, use of BPA as a synthetic estrogen was abandoned because of diethylstilbestrol (DES), which was determined to have more potent effects. DES was prescribed to millions of women for 30 years then after that it was reported as carcinogenic and caused reproductive effects in girls born from mothers taking DES during their pregnancy. These adverse effects lead to indicate that a chemical with resemble chemical structure and properties (figure 2-1) such as BPA may be have similar toxic effects (Rubin and Soto, 2009).In 1939, introduced in manufacture of epoxy resin (Plastic Historical Society, 2007) and in the 1940's, it began used in the plastic production (Wyatt, 2011). In 1993, by accident many of adverse effects of BPA on health were discovered when firstly report leaching of BPA from autoclave polycarbonate flasks, causing increased proliferation of breast cancer cells (Krishnan *et al.*, 1993).

2-1-2-Chemical structure of bisphenol-A

Bisphenol-A- is an organic compound have two equivalent phenol groups, in last decade, BPA classified as one of the endocrine disrupting chemicals as listed by (Ying and Kookana, 2002) because of its estrogenic properties (Sonnenschein and Soto, 1998).

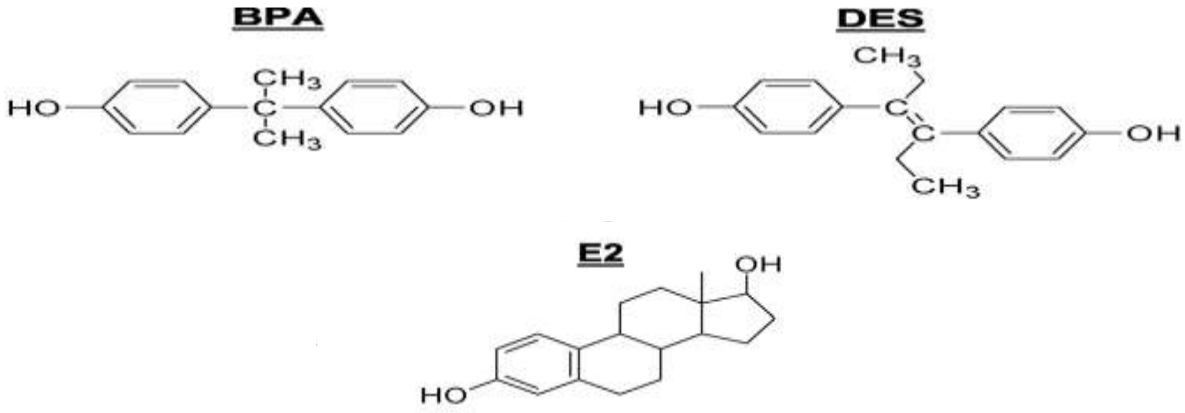

Fig.2-1. Structures of bisphenol A (BPA) and related compounds: estradiol (E2) and diethylstilbestrol (DES) (Wyatt, 2011)

2-1-3-Physical and chemical properties

Bisphenol A has common synonyms of [BPA or 2,2-bis (4-hydroxyphenyl) propane].BPA is characterized by its resistant to high temperatures, adhesive in nature and tough this lead to be highly useful in industry (Willhite *et al*., 2008). The presence of two unsaturated phenolic rings in the structure of BPA make resembles to diethylstilbestrol (Krishnan *et al*., 1993) BPA is solid and at room temperature and standard pressure convert it prills, flakes and crystals. Bisphenol A atmosphere breakdown rapidly by photooxidize in atmosphere (Cousins *et al*., 2002).

Production of BPA done by condensation between phenol and acetone, moderate solubility, high melting point is the main feature and low vapor pressure (Cousins *et al*., 2002 and Shareef *et al*., 2006). Low or moderate hydrophobicity (Heinonen *et al*., 2002 and Cousins *et al*., 2002).The

significance of solubility in environmental modeling may be illustrated by the calculation by Lai and co-workers (Lai *et al*., 2000).

2-1-4-Toxicity of BPA

The relatively high dose of estrogen or estrogenic compounds lead to serious problems in the fetus and neonate as well as with less degree in adult animals, the reproductive tract is fragile during the development stage, so that mostly subjected to irreversible effect after the exposure (Iguchi, 1992 and McLachlan, 2001).

In vitro studies have shown that BPA caused estrogenic effect by binding to the nuclear estrogen receptor (Bolger *et al.,* 1998; Nagel *et al.,* 1998 and Blair *et al.*, 2000). Experiments in rats and mice dosaged BPA orally lead to induction of clear estrogenic effects such as increased uterus weight at doses higher than 50 mg/kg (Ashby and Odum, 2004). Bisphenol A at doses of 10 mg/kg/day for two days showed influence on insulin secretion in mice (Alonso-Magdalena *et al*., 2006 and Alonso-Magdalena *et al*., 2008).

However, there is controversy if external exposure is really a good estimate for internal exposure due to differences in toxicokinetics of bisphenol A. So the blood concentrations of bisphenol A in humans are very important because toxicokinetics differences between species are accounted for when toxic effects are related to blood concentrations rather than to external exposure (Teeguarden *et al*., 2005).Toxicokinetic models have been used to simulate the bisphenol A kinetics in humans (Filser *et al*., 2003 and Teeguarden *et al*., 2005).

During developmental stage interference with gene expression regulation could occur as a direct or indirect consequence of exposure to these toxicants. Time of exposure, the dose, the nature of the compounds and period

of exposure effects the mechanisms causing the adverse effects on testicular development and function (Sharpe and Skakkebaek, 2008). Bisphenol A also can cause progressive effects in the oviduct and uterus (Savabieasfahani *et al*., 2006 and Newbold *et al*., 2007). Moreover, the expression level of estrogen receptor alpha (Esr1) and estrogen receptor beta (Esr2) increased in the uterus (Richter *et al*., 2007). Bisphenol A was reported to increases the oxidative stress, which is independent of estrogenic activity (Nakagawa and Tayama, 2000; Bindhumol *et al*., 2003 and Asahi *et al*., 2010).

2-1-5-BPA as endocrine disruptor

Bisphenol A is one of environmental endocrine disruptors that are released into the environment and have the ability to disrupt the normal functioning of the endocrine system in laboratory animals, humans and wildlife (Colborn *et al*., 1993).Estrogenic endocrine-disrupting chemicals typically have an affinity for estrogen receptors within the range of 1,000 to 100,000-times lower than estradiol (Welshons *et al*., 1999), and thus, are often described as being "weak" estrogens. While these chemicals are not as potent as estradiol, what is critical is the minimum or reference dose of estradiol capable of producing an effect during development (that is, the minimum change in serum estradiol relative to baseline levels *in vivo*) (vom Saal *et al*., 1997).

Based on endocrine disrupting actions of BPA as well as its structural similarity to thyroid hormone (TH), with its two benzoic rings, BPA has been proposed to act as a TH antagonist or TH agonist to cause disruption of thyroid system (Hiroi *et al*., 2006 and Jung *et al*., 2007). In amphibians, the action of BPA as a TH antagonist has been reported by Iwamuro *et al*., (2003).Bisphenol A also suppressed thyroid hormone receptor (TR) a and b

gene expression in peripheral tissues of larvae, including the tail both *in vivo* and *in vitro*. According to Goto *et al.*, (2006), R. rugosa tadpole tails displayed marked apoptotic features by triiodothyronine (T3) treatment, which was blocked by a simultaneous treatment with BPA. Gestational exposure to chemicals that mimic or inhibit hormone action, commonly known as endocrine-disrupting chemicals (EDCs), can interfere with the hypothalamicpituitary- gonadal (HPG) axis and lead to infertility or disease in adults.

2-1-6-Production and uses of BPA

Many countries throughout the world have large production capacities for BPA, especially Germany, Netherlands, USA and Japan. In the EU alone, in 1997/98, annual consumption of BPA was estimated at approximately 640,000 tonnes per year. Global production is reported to be increasing at about 7% per year, and to meet the increase in demand. Manufacture of polycarbonate exhausted about 65% of BPA produced and about 25% used in production of epoxy resin and the others 10% utilized in other products flame retardants (*European Chemical News*, 1999).

Therefore, BPA used in production wide variety of products like thermal (fax) paper, adhesives, compact disks, bullet resistant laminate, car parts, safety helmets, protective coatings, containers(including returnable milk and water bottles), food can linings, powder paints, polycarbonate bottles, plastic window and the sheathing of electrical and electronic parts (Staples,1996).

2-1-7-Exposure to BPA

Many reports has estimate BPA can be measured in humans in serum, amniotic fluid, urine, follicular fluid, umbilical cord, blood and placental

tissue. In some cases, the levels of total BPA (free and conjugated) in human blood and other fluids are higher than the concentrations that have been reported to stimulate number of molecular endpoints in cell culture *in vitro*, and appear to be within an order of magnitude of the levels of BPA in animal studies (Markey *et al*.,2001).

Levels of BPA were measured in human fluids and tissues in many developed countries of the world. BPA can be estimated in the majority of individuals in these countries. The levels of BPA in residents of less-developed countries, however, remain unknown (Sajiki *et al*., 1999), many studies using a variety of different techniques have measured unconjugated BPA concentrations in human serum. Several investigators have measured BPA levels in placental tissue and amniotic fluid (Engel *et al*., 2006).

Most studies have focused on exposure to BPA from dietary sources. In fact, some studies were determined BPA levels in foods, especially in cans that lining with resin. A few other potential sources of BPA exposure, namely drinking water, air and dust, have received far less attention. Using literature from contamination in the environment (water, air, soil) and food contamination (can surfaces, plastic containers), the daily human intake of BPA was estimated at less than 1 microgram/kg body weight/day (Kang *et al*., 2006).

Previous results from different groups studying the effects of brushing on BPA leaching, boiling, and washing. Sun *et al*., (2000) reported that BPA released from polycarbonate bottles, but not glass bottles, on their first use. According out results of BPA leaching, exposure of infants was estimated for period destended from birth through 3 months of age (Wong *et al*., 2005).

Bisphenol A in food-contact papers is not unexpected because BPA is often used as a developer in paper production (Vinggaard *et al*., 2000).

Olea *et al*., (1996) reviewed that the results obtained by the conditions that support BPA leaching from the coating of cans. They used cans from factories and done carefully controlled studies on the effects of time of heating, temperature of storage, time of storage, temperature of heating and other factors on the level of BPA leaching. Low levels of BPA were found in water stored in unheated cans, when cans were heated to 100 °C, temperature should used for the preservation of canned foods, the BPA concentrations were increased in water 1.7-55.4 times.

2-1-8-Effect of BPA on hematological parameters

White blood cells count was significantly increased in rat exposed to high dose of BPA (Nitschke *et al*., 1988).Other studies indicated that estrogen increases of interferon – gamma (IFN-γ) secretion from splenic lymphocytes, which play important role in controlling the function of all key immune cells (Karpuzogle-Sahin *et al*., 2001).Results of Sugita-Konishi *et al*., (2003) study revealed that BPA lead to migration of the neutrophils into the peritoneal cavity, but reduced infiltration activity, in addition, BPA decreased the population of lymphocytes and macrophages in the spleen and its accumulation in the infected foci. In Iraq, Almossawi (2013) reported signifigant increase in WBC in female and male offspring from mothers exposed to 250 mg/kg /BW of BPA during pre and postnatal life.

On the other hand, RBCs count and Hb levels were significantly decreased in the same study. RBCs count and Hb concentration were significantly decreased in rats exposed to BPA **(**AL–Farhaan, 2015). Pereira and Cesquni (2003) reported that there are significant decrease in RBCs count and Hb concentration, which referred to occurs of anemia because of decrease

the blood or immunological diseases and different infections and some of cancer types because of exposure to BPA, which considered as a carcinogenic.

2-1-9- Effect of BPA on biochemical parameters

2-1-9-1-Liver enzyme

Liver is the most sensitive organ senses to biochemical changes; therefore, its functions are greatly affected by pollutants and highly affect its serum enzymes level (Coughlin, 2011).The repeated exposure to the toxic compounds leading to an increase in hepatocytes and might cause histological changes in these cells such as rupture of the hepatocytes membranes. This resulting in release of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) enzymes from the cytoplasm of the destroyed cells into the blood stream, which explain the elevation of these enzymes (Farrell, 1995).

Alanine Aminotransferase (ALT) and Aspartat Aminotransferase (AST) levels were significantly increased in rats orally administrated BPA at dose 50mg/kg/B.W for four weeks (Korkmaz *et al*., 2010). Almossawi (2013) reported signifigant increase in ALT and AST levels at day 90 of age of female and male offspring from mothers exposed to 250 mg/kg /B.W. of BPA during pre and postnatal life.

2-1-9-2-Blood glucose

Liver has an important role in glucose metabolism. In the postprandial state, glucose reaches to the liver and is converted into glycogen and fatty acids or oxidized into carbon dioxide. In the fasting state, liver released glucose into the bloodstream via glycogenolysis and gluconeogenesis (Somm *et al*., 2009 and Jayashree *et al*., 2013).Al-Mossawi (2013) reported significant increase in glucose levels of male rat exposed to 50 and 250

mg/kg/B.W. of BPA during pre and postnatal stages of their life. Alonso-Magdalena *et al*., (2006) reported that there are significant increase in glucose level in the blood in animals treated with BPA.

2-1-10- Effect of BPA on immunological parameters

Bisphenol A can influence the immune system like estrogen, although there is a report demonstrating *in vitro* that xenoestrogen decreased substrate adherence capacity of antigen presenting cells including macrophages (Segura *et al*., 1999).Other study demonstrated that BPA affects the immune system of experimental animals, by diminishing its ability to enhance a protective response against infections (Kamimura *et al*., 2003). Tumor necrotic factor-α (TNF-α) is very important inflammatory cytokine (Qi and Pekala, 2000 and Ryden and Arner, 2007).It is play important role in destroy tumor cells, virus-infected cells and in killing cells that change to abnormal state as a result to tired or exposure chemical substances (Harvey and Ferrie,2011).

It is firstly synthesized as a membrane associated monomer, which is cleaved and released as a homotrimer. TNF_α produced by human adipose tissue does not cleaved, but still near the cells that synthesized it and acts as an autocrine/ paracrine factor (Mohamed-Ali *et al*., 1997). Bisphenol-A has role in augmenting immune response especially IgG and Th 1 response. Development of the immune system is very sensitive to endocrine disrupting compound (EDC) exposure, such as estrogenic chemicals (Yoshino *et al*., 2004).

Bisphenol A has a negative effect on the recruitment of macrophages into tissues, this is an important mechanism in innate host defense .Monocyte chemoattractant protein-1 (MCP-1), a member of the chemokine family, seems to have a role in recruiting blood monocytes to converted tissue

macrophages during innate immune responses. Many cells such as smooth muscle cells, vascular endothelial cells, fibroblast and macrophages produce MCP-1 as a response to stimuli from LPS, IL-1-β and TNF-α. BPA was shown to decrease MCP-1 levels in a dose-dependent manner in human breast cancer cell line MCF-7 that express ER (Inadera *et al.*, 2000).

2-1-11-Effect of BPA on hormones of reproductive

Mahoney and Padmanabhan (2010) suggested that the BPA have a mechanistic effect on the local regulatory circuits of hypothalamus and pituitary. BPA exert its effects via interfering with either ESRα or ESRβ or with both receptors that belong to the hypothalamic-pituitary-gonadal (HPG) axis (Adewale *et al*., 2009). AL–Farhaan (2015) reported that the increase in levels of LH hormone in females indicator on infertility because it's secretion interfere with hypothalamic pituitary gonadal axis. Wetherill *et al*., (2007) reported that there are significant decrease in concentration of FSH hormone because of (17*β*– estradiol) effect, which inhibit of gonadotropin secretion in the pituitary gland. Bisphenol A at high dose causes significant decrease in level of LH hormone because of BPA effect, which induce on decrease of LH hormone in pituitary gland and change of gonadotropin-releasing hormone (GnRH) because of BPA (Knobil & Hotchkiss, 1988).

2-1-12-Effect of BPA on histopathological changes in liver, kidney, ovary, uterus and spleen

Aughey *et al*., (1984) reported that BPA at high doses causes changes in histology of kidney tissue in rats, these changes involved necrosis in urinary tubules, bleeding intertubules and inflation of epithelium layer for glomerulus. Histopathological examination of kidneys tissue of the male rats treated with

50mg/k.g.b.w.daily BPA for 30 days demonstrated vacuolation of epithelial cells lining renal tubules figure, narrowing lumen sections, While male rats treated group with 100 mg/k.g BPA revealed vacoulation in glomerulus cell, massive hemorrhagic area and infiltration of the inflammatory tuft surrounding blood vessels. On the other hand sever vacuolation in glomerular capillary tuft, necrosis in the epithelial cells lining glomeruli and renal tubuli and atrophy in glomerular capillary tuft also showed sever vacuolation in renal tubular and necrosis in the epithelial cells lining renal tubuli and atrophy in renal tubular in male rats treated with 200mg/k.g.b.w. (Hussein, 2015).

Furukawa *et al.*, (1994) reported that BPA lead to multiple necrosis and atrophy of red pulp in spleen tissue of rat. Some studies showed that BPA at high doses causes changes in ovary including necrosis, bleeding, presence of the large numbers of atretic follicles and failed in ovulation these considered indication on toxic effect for BPA (El-Mesalamy, 2009). Decrease in estrogen receptors (ER-α) and decrease the epithelium layer for uterus were the main changes in rat exposed to high dose of BPA (Berger *et al*., 2010).

The high dose (250 mg/kg B.W.) of BPA in female rats causes histopathological changes in liver tissue include clear congestion of central vein, the hepatocytes were larger and flattened with clear enlarged pyknotic nuclei. In addition, liver appeared irregular irradiation structure and obvious sinusoidal spaces (AL –Mossawi, 2013). AL–Farhaan (2015) reported that histopathological changing in tissues of liver, kidney, ovary and uterus of rats treated with BPA.

2-2-Grape skin

Grapes (*Vitis vinifera*) is one of the most widely fruit crops that grown in the world (Poudel *et al.*, 2008).There are many of phenolic compounds isolated from the black grapes such as resveratrol, quercetin, gallic acid,

flavone, procyanidin, anthocyanin, catechin, epicatechin, flavonols, ellagic acid and myricetin are the major ones in the skins (En-Qin Xia *et al*., 2010).Black grapes promise as novel anti-inflammatory activity (Greenspan *et al*., 2005), antimicrobial agents (Brown *et al*., 2009), antimicrobial activity against *Escherichia coli* O157:H7 (Kim *et al*., 2009), anti-cancer properties (Mertens-Talcott *et al.,* 2006), inhibit UV-radiation induced peroxidation activity (Bagachi *et al*., 1997 and Dragsted, 1998), anti-viral, scavenge free radicals, antiarthritic, antiulcerative and prevent skin aging (Lakshmi *et al*., 2013).

2-2-1-History of grape

The history of grape is long and abundant during Greek and Roman civilizations, grapes were referred for their use in wine making. Presently, three main species of grapes are found: French hybrids, North American grapes (*Vitis labrusca* and *Vitis rotundifolia*) and European grapes (*Vitis vinifera*) .Moreover, it also classified into raisin grapes, wine grapes (used in viniculture) and table grapes. Grape fruit contain many nutrient elements, such as phytochemicals, edible fibers, carbohydrates, vitamins and minerals (Shrikhande, 2000 and Wada *et al*., 2007).

2-2-2-Phenolic antioxidants in grapes

The good effects of grape and its derived food products are related to a variety of bioactive components in grapes (Nadtochiy and Redman, 2011). Phenolic antioxidants such as phenolic acids, resveratrol, procyanidins, catechins, and anthocyanins are major group of bioactive components (Frayne, 1986). About 63–182 mg of the phenolic components are present in each100 grams of fresh grapes (Hogan *et al.,* 2009). While contain high

percentage (65–76%) in flavonoids. Anthocyanins are the major group of the flavonoids in red grapes (Hogan *et al.*, 2009). Major portion of grape phenolic antioxidants are found in grape skins or seeds (Careri *et al.*, 2003).

Resveratrols, catechins, and antochyanins are concentrated in the skin of grape; on the other hand, procyanidins are concentrated in seeds of grape (Kammerer *et al.*, 2004).The source of commercial grape skin or seed extracts are grape pomace, which is the waste by product of wine making industry (Lu and Yeap *et al.*, 1999).Large amounts of pomace accumulate each year, which causes a waste-management issue (Bustamante *et al*., 2008). About 20% of harvested grape produce grape pomace. Moreover, grape pomace is limited use but may be recycled as animal food, organic fertilizers and manure (Hogan *et al*., 2010). Grape skins and seeds are the major constituents in the pomace, so its considered important source of phenolic antioxidants (Lu and Yeap *et al*., 1999 and Kammerer *et al*., 2004).

2-2-3-Effect of grape skin on hematological parameters

Al Jeboory *et al*., (2012) showed that there was an increase in Hb values and RBCs count in mice treated with proanthocyanidin which is one of components for grape seed and skin, while they demonstrated that there is decrease in WBCs counts in mice treated with proanthocyanidin. The results of neutrophils% showed a significant increase female rabbits treated with (Proanthocyanidin at a dose 200) compared with (+ve) control group, (-ve) control group and another treated groups while results of lymphocyte % showed a significant decrease of female rabbits treated with (Proanthocyanidin at a dose 100 and 200) compared with (+ve) control group (Al-Razaq, 2015).

2-2-4- Effect of grape skin on biochemical parameters

One of the major compounds for grape skin, is resveratrol when added to fed of rats causes decrease AST and ALT activities (Hamadi *et al.*, 2012).

Adisakwattanana *et al*., (2010) reported decrease in the serum glucose level in rats administrated proanthocyanidin (one of component major of grapes) orally and suggest that these effects were related to inhibition intestinal α-glucosidase and pancreatic α-amylase.

2-2-5- Effect of grape skin on immunological parameters

Treatment with resveratrol which is one of the main components for grape skin showed decrease in level of IgG in serum of rats after 2, 4 and 6 weeks (Omayma *et al* ., 2015).Results of Greenspan *et al.*, (2005) showed that the more dilute solution of muscadine grape extract (1:400) lead to inhibition of IL-1β production by approximately 60%. Diluted its also inhibited production of TNF-α and IL-6 by nearly 50%. These results indicated that some constitutes component of muscadine grape skin extract may have anti-inflammatory properties.

2-2-6- Effect of grape skin on hormones of reproductive

There are many transduction pathways, which included in the effects of resveratrol on cell functions such as reduce proliferation of theca-interstitial cells by inhibition of the mevalonate pathway (Wong *et al*., 2011). Khattab *et al*., (2010) who indicated that grape seed extract alleviate reproductive toxicity caused by aluminum chloride in male rats. LH and FSH concentration a significant increase of treated groups with Proanthocyanidin at a dose 100mg/kg and200 mg/kg) respectively compared with (–ve control) group. On the other hand, estradiol concentration was a significant decrease in serum of female

rabbits treated with Proanthocyanidin at a dose 100mg/kg and200 mg/kg) respectively (Al-Razaq, 2015).

2-2-7-Effect of grape skin on histopathological changes in liver, kidney, ovary, uterus and spleen

Ray *et al*., (1999) who showed that grape skin powder extract in liver tissue significantly decreased acetaminophen-induced lethality, positively influence gene expression in mice, ALT activity, hepatic DNA damage, apoptotic cell death and liver toxicity. Al-Razaq (2015) reported that administration (100mg\kg and 200mg\kg) of proanthocyanidine lead to healing of renal damage due to its properties as antioxidant and free radical scavenging .In the same study secondary follicles (SF), normal architecture, normal primary follicles (PF) and normal ovarian cellular tissue with normal graafiane follicles (GF) in ovary of female rabbits orally administrated (100 and 200 mg\kg B.W.) of proanthocyanidin. In addition the uterus of these female rabbits orally administrated (100 and 200 mg\kg B.W.) of proanthocyanidin appeared dilated lumen, normal architecture, papillary epithelium, normal endomaterium (proliferative phase), normal uterine glands (UG) and amelioration of uterine tissue. Resveratrol, the main component of grape skin have protective effects on spleen tissue in rats due to it multitargeted agent of resveratrol *(*Karabulut *et al*., 2006).

CHAPTER THREE

MATERIALS AND METHODS

3- Materials and Methods

3-1- Materials

3-1-1-Animals of the study

The present study was conducted in animal house of College of Veterinary Medicine – University of Karbala. Forty mature female (*Rattus norvegicus*) rats were purchased from the care center and medicinal researches in Baghdad, Iraq. They were 14 to 16 weeks old with an average body weight (200-250gm).The animals were clinically healthy, kept under hygienic conditions, metal cages and glassy bottles were used to avoid exposure to BPA from old polycarbonate cages. Water and food were giving *ad –libitum* throughout the experimental period. The animals were left in the laboratory conditions for 2 weeks before beginning of experiment to be accommodated. The light system was 12/12 hrs light/dark cycle.

3-1-2-Laboratory chemicals, instruments and kits were used in this experiment are represented in tables (3-1), (3-2) and (3-3) with their suppliers respectively.

Table (3-1) shows chemicals and their suppliers

Chemicals	**Suppliers**
Bisphenol A (CAS 80-05-7 > 99% pure)	Sigma Aldrich Company (USA).
Chloroform	Merk, Germany
Eosin stain	Merck, Germany
Ethanol	Merk, Germany
Formalin40%	TEDIA company. USA
Hematoxylin stain	Merck, Germany
Methanol	GCC. England

Na_2HPO_4	Thomas Baker/USA
NaH_2PO4	Thomas Baker/USA
Paraffin wax	Merck, Germany
Sodium Chloride (NaCl)	Thomas Baker/USA
Tocopherol-stripped corn oil	ICN Biomedicals Inc.USA
Xylole	Scharlau, Spain

Table (3-2) shows instruments with their suppliers and sources.

Instruments	**Suppliers**	**Sources**
Autoclave	YX-280 B	China
Camera Digital	Sony	Japan
Centrifuge	Hettich Roto fix11	Japan
Curve scissor	Pakistan	Pakistan
Digital Vernia Calipers	Metrology Inc.	India
Distiller	Labtech	korea
Electronic Balance	Metter company	Switzerland
Eppendrof tubes	Merk	Germany
Graduated Tube	Merk	India
Hot plate	Alssco	India
Incubator	Fisher Scientific	Germany
Light microscope	Olympus	Japan
Micropipette	Human company	Germany
Microplate Elisa Washer	Human	Germany
Microplate Elisa Reader	Human	Germany
Milipore filter unite	Gallenkamp	England
Multiple Pipette	Slamed	Germany

Optical reader	Cleaver scientific	Japan
Oven	Memmert	Germany
PH-meter	Mauritius	Germany
Printer-micro ELISA	Human	Germany
Refrigerator	Concord	Italy
Rotary Macrotome	Human	Germany
Semi-auto chemistry analyzer	Genex x Ceem-S1	USA
Sensitive balance Mlter AE 2000	Sartouris	Germany
Spectrophotometer	Tudor	Korea
Vet. Hematological auto analyzer	Genex 60 Vet	USA
Vortex shaker	Griffin	Germany
Water bath	K.F.T LAB.Equipment	Italy

Table (3-3) shows kits with their suppliers.

ALT (GPT) Colorimetric .Kit	Spectrum company, Egypt
AST (GOT) Colorimetric .Kit	Spectrum company, Egypt
Complement proteins (C_3)	Easy RID(Italy)
Estradiol (E2) ELISA Kit	Monobind Inc. USA
Follicle Stimulating Hormone (FSH) ELISA Kit	Monobind Inc. USA
Glucose Colorimetric .Kit	Spectrum company, Egypt
Immunoglobulines IgG	Easy RID(Italy)

Luteinizing Hormone (LH) ELISA Kit	Monobind Inc. USA
Rats Interleukin(IL-1β)ELISA Kit	Bostebiological technology Co.,Ltd
Rats Interleukin(IL-2)ELISA Kit	Bostebiological technology Co.,Ltd
Rats Interleukin(IL-6)ELISA Kit	Bostebiological technology Co.,Ltd
Rats TNF-α ELISA Kit	Bostebiological technology Co.,Ltd

3-1-3-Ration formula

The compositions of ration used in the study are summarized in table 3-4.

Table (3-4) shows components of one kilogram of diet

Material	**Amount**
Corn	250 gram
Crude protein	180 gram
Minerals and vitamins	1 gram (additives)
Powder milk	20 gram
Vegetable oil	20 gram
Wheat	530gram

50 grams of grape skin powder were added to the ration for each 1Kg to third group and fifth group of the present study.

3-2-Methods

3-2-1-Preparation of Bisphenol –A- (BPA)

Bisphenol A (BPA, CAS 80-05-7, > 99% pure) was imported from Sigma Aldrich Company (USA) (Chang *et al.* 1994).Tocopherol-stripped corn oil (ICN Biomedicals Inc., USA) served as the vehicle and control substance. BPA were mixed with corn oil to get the desired concentration (250

mg/kg B.W of BPA). Fresh suspension were prepared every week and stored in glass containers. Based on the body weight of female rats dose was administered orally to each one.

3-2-2-Preparation of grape skin powder

Red grapes (*Vitis Labruscana Bailey*) were obtained from Korea and the following procedure done according to (Shin and Moon ,2010)

Procedure of the preparation

1-One hundred kg of red grapes were used to prepare 1kg of grape skin powder.

2-The grapes were washed and air dried at room temperature on a paper towel and the stems were removed before their peeling.

3-Grape skins were peeled and removed about other grape components such as grape seeds and pulps by using sharp tool.

4-Peeled grape skins were dried by using oven at 70C for several hours.

5-Dried grape skins were mashed by using iron mortar until grape skins conversed to small molecules like powder.

6- One kilogram of grape skin powder were added to 20 kg of food animals to third and fifth groups for 30 days.

3-3-The experimental design

The experiment extends for 30 days from 13-12-2015 until 11-1-2015. Forty female albino rats were divided into five main groups (8animals) of each group as following:

1-General control (GC) group: Eight female rats that received only normal saline orally as vehicle (0.5ml/kg B.W.).They were offered normal ration.

2-Vehicle control (VC) group: Eight female rats that received only corn oil orally as vehicle (0.5ml/kg B.W.). They were offered normal ration.

3-Treatment 1(T1) group: Eight female rats that received only diet mixed with 5% grape skin powder orally as ration. Water was supplied ad libitum.

4-Treatment 2(T2) group: Eight female albino rats, orally administered BPA 250 mg/kg B.W. /day (1/20 LD50) suspended in corn oil via gavage as high dose (NIOSH, 1978).Normal ration and water were supplied ad libitum.

5- Treatment 3 (T3) group: Eight female albino rats, orally administered BPA 250 mg/kg B.W. /day (1/20 LD50) suspended in corn oil via gavage as high dose (NIOSH, 1978).Diet mixed with 5% grape skin powder and water were supplied ad libitum.

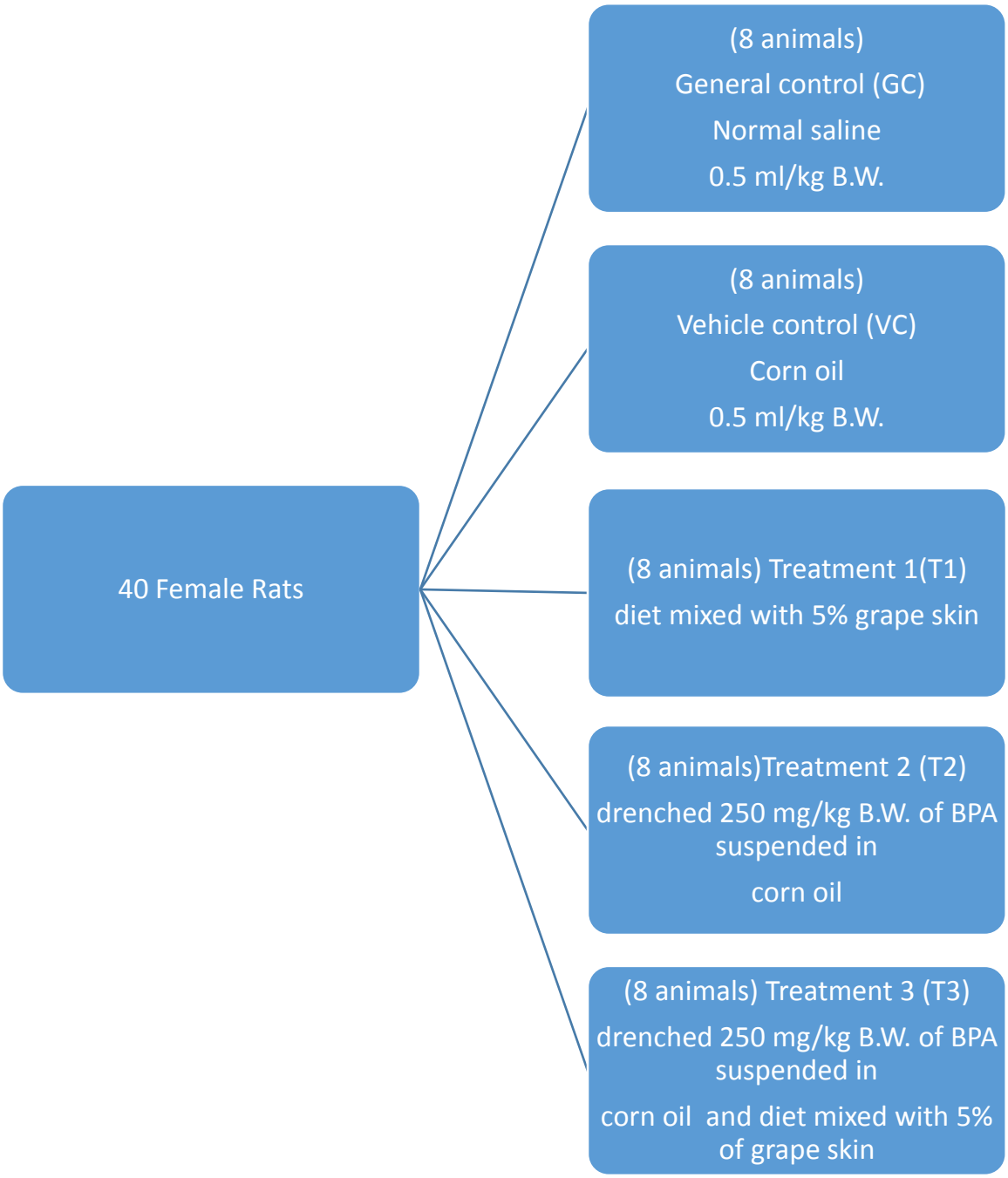

Figure (3-1) Experimental design

3-3-1-Blood samples collection

Blood samples were collected by heart puncture. Then the blood sample were dropped directly from the heart by using (5ml) disposable syringe (1ml) of blood collected in heparinized tube for hematological parameters which were measured as soon as possible.

The rest of the blood was put in plane tube to be centrifuged (6000 rpm for 10 minutes) to obtain the serum, which is then transferred to three epndrofe tubes, the first one is used for hormones measurement, the second for the immunological estimation and the last one for the rest biochemical tests. All tubes were stored at (-20c) until analyzed.

3-3-2-Organs samples collection

Eight female rats' of each group were sacrificed at the end of the experiment after 30 days. The rats before that were first anaesthetized by placing them in a closed jar containing cotton sucked with chloroform anesthesia. Kidney, liver, ovary, uterus and spleen were isolated and trimmed of their fat. The organs were fixed in 10 % formalin for histological examination.

3-4-Parameters of study

3-4-1-Hematological parameters

Hematological Parameters were done by using Veterinary automated hematoanalyzer (Genex Inc., Florida USA).The blood parameters were included: Total and differential Leukocytes count(lymphocyte, monocyte, neutrophil, eosinophil and basophil), Hemoglobin and Total Erythrocytes count.

3-4-2-Biochemical parameters:

3-4-2-1-Determination of serum aspartate aminotransferase level

Serum aspartate aminotransferase level (AST) is determined by using a special kit (SPECTRUM AST – kit, Egypt) (Young, 1990)

Principle

Colorimetric determination of AST level was performed according to the following reactions:

AST: Aspartate + α keto glutarate $\xrightleftharpoons{\text{AST}}$ oxaloacetate + glutamate

The reaction:

The oxaloacetate formed is measured in its derivative form, 2,4-dinitrophenylhydrazone.

Reagents

Reagent 1	Phosphate buffer pH7.5	100 mmol / l
AST	L-Aspartate	100 mmol / l
	2-Oxoglutarate	5 mmol / l
	Sodium Hydroxide	140mmol/l
	SodiumAzide	12 mmol/l
Reagent 2	2.4dinitrophenylhydrazine	2 mmol / l
Color reagent	HCL	8.4 %

Procedure

Wave length: 546 nm (530 – 550 nm)

Zero adjustment: reagent blank:

Pipette into test tubes:

Reagents	Reagent blank	Sample
Reagent 1(Buffer)	0.5 ml	0.5 ml
Sample	------	100µl
Distilled water	100µl	------
Mix and incubate for exactly 30 minutes at 37^0C		
Reagent 2	0.5 ml	0.5 ml
Mix and incubate for exactly 20 minutes at $20\text{-}25^0$C		
Sodium hydroxide	5 ml	5 ml
Mix and measure absorbance of specimen against reagent blank at 546nm after 5 minutes		

Calculation

Obtain the AST level from the following table

Absorbance	Value of AST U/L	Absorbance	Value of AST U/L
0.020	7	0.100	36
0.030	10	0.110	41
0.040	13	0.120	47
0.050	16	0.130	52
0.060	19	0.140	59
0.070	23	0.150	67
0.080	27	0.160	76

0.090	31	0.170	89

Linearity: the assay is linear up to 89 U/L.if the absorbance exceeds 0.170 at 546nm , sample should be diluted 1+9 using sodium chloride and repeat the assay (result x 10).

3-4-2-2-Determination of serum alanine aminotransferase level

Serum alanine aminotransferase level ALT is determined by using a special kit (SPECTRUM ALT – kit, Egypt) (Young, 1990)

Principle

Colorimetric determination of ALT level was performed according to the following reactions:

$$\text{ALT : Alanine} + \alpha\text{ keto glutarate} \xrightleftharpoons{\text{ALT}} \text{pyruvate} + \text{glutamate}$$

The pyruvate formed is measured in its derivative form, 2,4-dinitrophenylhydrazone .

Reagents

Reagent 1 **ALT**	Phosphate buffer pH7.5 D-Alanine 2-Oxoglutarate SodiumAzide	100 mmol / l 200 mmol / l 6 mmol / l 12 mmol/l
Reagent 2 **Color reagent**	2.4dinitrophenylhydrazine	2 mmol / l

Procedure

Wave length: 546 nm (530 – 550 nm)

Zero adjustment: reagent blank:

Pipette into test tubes:

Reagents	Reagent blank	Sample
Reagent 1(Buffer)	0.5 ml	0.5 ml
Sample	----	100µl
Distilled water	100µl	----
Mix and incubate for exactly 30 minutes at 37^{0}C		
Reagent 2	0.5 ml	0.5 ml
Mix and incubate for exactly 20 minutes at 20-25^{0}C		
Sodium hydroxide	5 ml	5 ml
Mix and measure absorbance of specimen against reagent blank at 546nm after 5 minutes		

Calculation

Obtain the ALT level from the following table.

Absorbance	Value of AST U/L	Absorbance	Value of AST U/L
0.025	4	0.275	48
0.050	8	0.300	52
0.075	12	0.325	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77
0.200	34	0.450	83

0.225	39	0.475	88
0.250	43	0.500	94

Linearity: the assay is linear up to 94 U/L.if the absorbance exceeds 0.5 at 546nm, sample should be diluted 1+9 using sodium chloride and repeat the assay (result x 10).

3-4-2-3-Estimation of glucose

Serum glucose was enzymatically measured by using a linear chemical kit (RANDOX\GLUC-PAP, United Kingdom) (Alonso-Magdalena *et al*.,2010).

Principle of test:

The principle of these measurement is existing as the following equation:

Glucose $+O_2 + H_2O \xrightarrow{GOD}$ gluconic acid + H_2O_2

$2\ H_2O_2$ + 4-aminophenazone +phenol $\xrightarrow{POD}$ quinonemine + $4H_2O$

Procedure:

The procedure of this kit as the following:

Solution	Blank	Standard	Sample
Reagent	1000µl	1000µl	1000µl
Standard	-	10µl	-
Serum	-	-	10µl

Mix, incubate for 25 min. at 15-25c° or 10 min. at 37c°. Then measured absorbance of sample and standard against reagent blank at 546 nm within 60 min.

The formula of serum glucose concentration was

$$\text{Serum glucose concentration (mg\dl)} = \frac{\text{A sample}}{\text{A standard}} \times \text{n}$$

Where n = 100mg \dl concentration of standard solution.

3-4-3-Immunological parameters

3-4-3-1-Interleukines

3-4-3-1-1-Interleukin (IL-1β)

Assay procedure

1-The ABC working solution and TMP color developing agent were kept warm at 37 ºC for 30 min before use.

2-When diluting samples and reagents, they were mixed totally and equally.
3-Standard IL-1β detection curve were prepared for each experiment, the user will decide what will be folded by crude estimation of IL-1β amount in samples.

4- Add (0.1ml) per well of the 800pg/ml, 400pg/ml, 200pg/ml, 100pg/ml, 50pg/ml, 25pg/ml,12.5pg/ml rat IL-1β standard solutions into the precoated 96-well plate.

5-Add (0.1ml) of the samples diluent buffer into the control well (zero well).

6- Diluted sample serum (0.1) was added to each well.

7- The plate was sealed with the cover and incubate at 37 ºC for 90 min.

8- The cover was removed, plate content was discarded and the plate was blotted into paper towels.

9-Biotinylated anti-rat IL-1β antibody working solution (0.1ml) was added into each well and incubated the plate at 37 ºC for 60 min.

10- Plate was washed three times with (0.01M) PBS, and each time washing buffer stayed in the wells for 1 min and repeated two additional times.

11-The washing buffer was discarded and the plate was blotted onto paper towels.

12-Prepared ABC working solution (0.1 ml) was added into each well and incubated the plate at 37ºC for 30 min.

13- Plate was washed (5 times) with (0.01M) PBS, and each time washing buffer stayed in the wells for 1 min.

14- Washing buffer was discarded and the plate was blotted onto paper towels.

15- Prepared TMP color developing agent (90 µl) was added into each well and incubated plate at 37ºC in dark for 25-30 min.

16- Prepared TMB stop solution (0.1 ml) was added into each well. The color changes into yellow directly.

17- Determining of absorbance (OD) of each well was done at (450 nm) by microplate reader.

3-4-3-1-2-Interleukin (IL-2)

Assay procedure

Procedure of the test similar to that of (IL-1β) except add (0.1ml) per well of the1000pg/ml, 500pg/ml ,250 pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml, 15.6pg/ml rat IL-2 standard solutions in replace of add (0.1ml) per well of

the 800pg/ml, 400pg/ml, 200pg/ml,100pg/ml, 50pg/ml,25pg/ml,12.5pg/ml rat IL-1β standard solutions.

3-4-3-1-3-Interleukin (IL-6)

Assay procedure

Procedure of the test similar to that of (IL-1β) except add (0.1ml) per well of the1000pg/ml, 500pg/ml ,250 pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml, 15.6pg/ml rat IL-6 standard solutions in replace of add (0.1ml) per well of the 800pg/ml, 400pg/ml, 200pg/ml,100pg/ml, 50pg/ml,25pg/ml,12.5pg/ml rat IL-1β standard solutions.

3-4-3-1-4-Tumer Necrotic Factor-α (TNF-α)

Assay procedure

Procedure of the test similar to (IL-1β) except add (0.1ml) per well of the1000pg/ml, 500pg/ml ,250 pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml, 15.6pg/ml rat TNF-α standard solutions in replace of add (0.1ml) per well of the 800pg/ml, 400pg/ml, 200pg/ml,100pg/ml, 50pg/ml, 25pg/ml, 12.5pg/ml rat IL-1β standard solutions.

3-4-3-2-Complement proteins (C_3)

Principle of the method

EASY RID permits the determination of human plasmaproteins in single radial immunodiffusion. The antigen (protein) was added in the well on the plate, spreads radially in the agarose gel, reacting with antibodies incorporated in agarose gel and creating immune complexes visible as precipitin rings. Diameter of precipitin ring is directly proportional to the concentration of the relevant protein in the sample (Berne, 1974).

Test procedure

1- EASY RID was removed from the envelope, the plate was opened and left to stand for about (5minutes) at room temperature in order to evaporate any condensed water in the wells.

2- Undiluted serum of samples (5µl) was put into wells.

3- The plate was closed with the lid, after the samples have diffused into the gel, left to stand, overturned into the envelope, at room temperature for (48 hours).

4-Diameter of the precipitin rings was measured after (48 hours) by using appropriate device (optical reader) (mm).the protein concentration for the precipitin ring diameter was read by using the attached tables.

3-4-3-3-Immunoglobiolines (IgG)

Principle of the method

Principle of IgG similar to that of C3 (Berne, 1974)

Test procedure

Test procedure similar to that of C3

3-4-4-Hormonal Assay (Enzyme-Linked Immunosorbent Assay "ELISA")

The basic principle of an Enzyme linked immunosorbent is to use an enzyme to discover the binding of antigen (Ag) antibody (Ab). The enzyme changes a colorless substrate to a colored product, showing the existence of Ag: Ab binding (Ma *et al.*, 2006).

3-4-4-1-Estimation of Follicle Stimulating Hormone (FSH) concentration (µlU/ml)

Determination of serum Follicule Stimulating Hormone concentration by using specific kit (Monobind Inc. lake forest CA 92630, USA).

Principle of the test

The Monobind (FSH) ELISA is based on the principle of competitive enzyme immunoassay; the necessary reagents required for a solid stage enzyme immunoassay include native antigen, enzyme-antigen conjugate and immobilized antibody.

Procedure of the test

The test procedure was conducted according to the following stages:

1-(1) ml of FSH enzyme conjugate was diluted with (11ml) of total FSH conjugate buffer in appropriate container to prepare FSH-enzyme conjugate solution .

2-(20) ml of concentrated washing solution was prepared and then was diluted with (980ml) of distilled water to final volume of (1000ml).

3- Solution (A) was mixed with solution (B) to prepare substrate solution.

4- Required number of microplate wells were secured in the holder.

5- Standard solution (50µl) and serum of experiment groups were put into the marked wells (all samples were run in duplicate at same time to keep same conditions of testing).

6- FSH enzyme conjugate solution (100µl) was put to each well.

7- Gently mixing of microplate was done for 20-30 seconds.

8- Incubation of microplate was extend (1hour) at room temperature.

9- Drawn of wells contents was done by manual plate washer, then washed three times with prepared washing solution (300µl per well).

10- Substrate solution (100 µl) was added to each well.

11- Incubation of microplate was done by 15 minutes at room temperature.

12- Stop solution (50µl) was added to each well in order to stop enzymatic reaction.

13- Determining the absorbance (OD) of each well was done at wave length (450nm) by microplate reader.

3-4-4-2-Estimation of Luteinizing Hormone (LH) concentration (ng/ml)

Determination of serum gonadotropin (LH) concentration by using specific kit (Monobind Inc. lake forest CA 92630, USA).

Principle of the test

The Monobind (LH) ELISA is depended on the principle of competitive enzyme immunoassay; the necessary reagents needed for a solid stage enzyme immunoassay includes native antigen, enzyme-antigen conjugates and immobilized antibody.

Procedure of the test

The procedure of the test is similar to that of FSH except of adding LH enzyme conjugate solution.

3-4-4-3-Estimation of Estradiol (E2) concentration (pg/ml)

Determination of serum concentration is normally considered as valued instrument in the analysis of the growth of ovarian follicular; Kit was used (Monobind Inc. lake forest CA 92630, USA).

Procedure of the test

The test procedure was done according to the following stages:

1- Required number of microplate wells were secured in the holder.

2- Standard solution (50µl) and serum of experiment groups were put into the marked wells (all samples were run in duplicate at same time to keep same conditions of testing).

3- Estradiol Biotin Reagent (50µl) was put into each well.

4- Gently mixing of microplate was done for 20-30 seconds.

5- Incubation of microplate was extended half hour at room temperature.

6-Estradiol Enzyme Reagent (50µl) was put in to each well.

7- Gently mixing of microplate was done for 20-30 seconds.

8- Incubation of microplate was done for (90 minutes) at room temperature.

9-Drawn of wells contents was done by manual plate washer, then washed three times with prepared washing solution (300µl per well).

10-Substrate solution (100µl) was added to each well.

11-Microplate was incubated for (20 minutes) at room temperature.

12-Stop solution (50µl) was added to each well in order to stop the enzymatic reaction.

13- Determining the absorbance (OD) of each well was done wave length at (450 nm) by microplate reader.

3-4-5-Histopathological technique

Liver, kidneys, ovary, uterus and spleen of each animal were rapidly removed, then prepared for histological estimate according to Mescher method (2010).

These organs were fixed in 10% buffered formalin, dehydrated progressively in increased ethanol concentrations, treated with xylene and embedded in paraffin. Five microns thickness sections of paraffin-embedded tissue were mounted on glass slides rehydrated progressively in decreased ethanol concentrations and stained with Hematoxyline and Eosin stain (H &

E stain)(Bancroft *et al*.,1990 and Luna,1968) and then estimated with help of the light microscope.

3-5-Statistical analysis

The data were presented as mean ±SE and subjected to analysis of variance by using one way ANOVA Post hoc test was used LSD to specify the significant difference among means the software package IBM SPSS Program version 20 was used for the analysis of data (2001).

CHAPTER FOUR

RESULTS

4-Results

4-1- Effect of BPA and grape skin on some hematological parameters in mature female rats

4-1-1-The effect of BPA and grape skin on total and percentage of differential count of WBC in mature female rats

Blood parameters in table (4-1-1) were revealed significant increase ($p \leq 0.05$) in WBC count of animals in a group treated with 250 mg/kg/B.W /day of BPA compared with all other groups of study. While, statistical analysis offered there were significant decrease ($p \leq 0.05$) in WBCs count in group of BPA and their diet mixed with grape skin. Nevertheless, there were non-significant changes ($p \geq 0.05$) in WBC count in the remaining four groups of study. Other results also showed enhancement in level of WBCs count in two groups of BPA and their diet mixed with grape skin and grape skin.

The percentage of lymphocyte in the present study showed a significant decrease ($P \leq 0.05$) in female rats treated with 250 mg/kg/B.W/day of BPA compared with all other groups. In contrast, there was significant increase ($p \leq 0.05$) in lymphocyte % in group exposed to BPA and their diet mixed with grape skin although not reach to the normal rate of control groups as compared with (T2) that received BPA alone. Nevertheless, there were non-significant changes ($p \geq 0.05$) in lymphocyte % between group of grape skin and control groups.

The percentage of monocyte, eosinophil and basophil was non-significantly differ ($p \geq 0.05$) in all treated groups. While, the result of neutrophils % showed a significant increase ($P \leq 0.05$) in female rats exposed with 250 mg/kg/B.W /day of BPA compared with all other groups. Whereas, significant decrease ($p \leq 0.05$) was noted in neutrophils % in group exposed to

BPA and their diet mixed with grape skin compared with group of BPA only. However, there were non-significant changes ($p \geq 0.05$) in neutrophils % in group of grape skin and control groups.

4-1-2-Effect of BPA and grape skin on RBCs count and Hb concentration in mature female rats

A significant decreases ($p \leq 0.05$) in RBCs count and Hb level were appeared in group that exposed to 250 mg/kg/B.W /day of BPA in comparison with all other groups of study in table (4-1-2). While the group administrated BPA and their diet was mixed with grape skin showed significant raise ($p \leq 0.05$) in RBCs count and Hb level in contrast with BPA.

Table (4-1-1) the effect of BPA and grape skin on total and percentage of differential count of WBC in mature female rats (Means ± SE)

Parameters / Groups	WBC $\times 10^3$cell/mm^3	Lymphocyte %	Monocyte %	Neutrophil %	Eosinophil %	Basophil %
Normal saline group General control (GC) group (0.5ml/kg/B.W)	B 8.15±0.16	A 89.57±0.61	A 1.50±0.21	C 6.85±0.55	A 1.64±0.17	A 0.42±0.17
Corn oil group Vehicle control (VC) group (0.5ml/kg/B.W)	B 8.27±0.22	A 89.87±0.39	A 1.37±0.18	C 7.37±0.91	A 1.12±0.24	A 0.25±0.09
Grape skin group Treatment 1 (T1) group (5%mixed with feed)	B 8.09±0.50	A 89.12±0.63	A 1.37±0.15	C 8.50±0.50	A 0.75±0.18	A 0.25±0.09
Bisphenol-A- group Treatment 2 (T2) group (250 mg/kg/B.W)	A 11.66±0.33	C 72.37±1.86	A 1.25±0.09	A 24.25±1.86	A 1.50±0.16	A 0.62±0.15
Bisphenol-A-and Grape skin group Treatment 3 (T3) group (250 mg/kg/B.W of BPA +5% grape skin mixed with feed)	B 8.70±0.37	B 84.50±1.14	A 1.25±0.16	B 12.75±0.88	A 1.25±0.21	A 0.37±0.15

N=8

Different letters represent a significant difference at ($p \leq 0.05$)

Table (4-1-2) the effect of BPA and grape skin on RBCs count and Hb concentration in mature female rats (Means ± SE)

Parameters / Groups	RBC $\times 10^6$cell/mm^3	Hb g/dl
Normal saline group General control (GC) group (0.5ml/kg/B.W.)	A 6.73±0.19	A 13.71±O.42
Corn oil group Vehicle control (VC) group (0.5ml/kg/B.W.)	B 5.76±0.21	A 13.25±O.45
Grape skin group Treatment 1 (T1) group (5% mixed with food)	A 6.83±0.28	A 13.37±0.67
Bisphenol-A- group Treatment 2 (T2) group (250 mg/kg/B.W.)	C 4.16±0.21	B 8.00±0.46
Bisphenol-A-and grape skin group Treatment 3 (T3) group (250 mg/kg/B.W. of BPA +5% grape skin mixed with food)	B 5.89±0.23	A 12.62±0.37

N=8

Different letters represent a significant difference at (p≤0. 05)

4-2- Effect of BPA and grape skin on some serum biochemical parameters in mature female rats

Serum ALT level of female rats treated with 250 mg /kg B.W./day of BPA exhibited significant increase ($p \leq 0.05$) when compared with female rats that fed with grape skin powder and with that drenched corn oil or normal saline. While, group that received 250 mg /kg B.W./day of BPA and their diet mixed with grape skin demonstrated significant enhancement ($p \leq 0.05$)in mean of ALT level against the group treated with grape skin only although not reach to the normal rate of control groups.

The analysis of variance in the present study found out significant rise ($p \leq 0.05$) in means of AST in group treated with 250 mg/kg B.W/day of BPA compared with all other groups. Moreover, the rats that their diet mixed with grape skin and administered 250 mg /kg B.W./day of BPA were exhibited significant reduce ($p \leq 0.05$) in levels of AST in contrast with the group which their diet was mixed with grape skin only while not belong to the level of control groups.

In addition, data of this study revealed a significant increase ($p \leq 0.05$) in serum glucose level in mature female rat exposed to 250 mg /kg B.W/day of BPA when compared with all other studied groups (table4-2). On the other hand, the value of serum glucose showed significant decrease ($p \leq 0.05$) in group treated with 250 mg /kg B.W. /day of BPA and their diet mixed with grape skin comparing with (T2) group. Female rats fed with grape skin only appeared significantly lowest ($p \leq 0.05$) in serum glucose level (table 4-2) as compared with (VC) group only.

Table (4-2) the effect of BPA and grape skin on Some serum biochemical parameters in mature female rats (Means ± SE)

parameters / Groups	ALT U/ml	AST U/ml	Glucose g/dl
Normal saline group General control (GC) group (0.5ml/kg/B.W.)	CD 47.71±1.04	C 80.28±1.01	BC 65.71±1.04
Corn oil group Vehicle control (VC) group (0.5ml/kg/B.W.)	C 52.62±2.57	C 98.75±2.68	B 69.37±1.82
Grape skin group Treatment 1 (T1) group (5%mixed with food)	D 43.50±1.28	C 91.12±2.40	C 60.87±1.25
Bisphenol-A- group Treatment 2 (T2) group (250 mg/kg/B.W.)	A 96.00±2.23	A 331.37±13.25	A 80.50±3.47
Bisphenol-A-and grape skin group Treatment 3 (T3) group (250 mg/kg/B.W. of BPA +5% grape skin mixed with food)	B 68.00±2.63	B 204.00±14.27	BC 64.75±4.02

N=8

Different letters represent a significant difference at (p≤0. 05)

4-3- Effect of BPA and grape skin on some serum immunological parameters in mature female rats

A significant increase ($p \leq 0.05$) was noticed in serum C_3 and IgG concentrations of female rats treated with BPA 250 mg/kg B.W. compared with all other groups and there was significant amelioration ($p \leq 0.05$) in the group exposed to BPA and their diet mixed with grape skin group compared with group of grape skin only and control groups but it was insignificant alterations ($p \geq 0.05$) between grape skin group alone and control groups.

Regarding to the interleukins BPA there was highly significant ($p \leq 0.05$) increase in serum IL-1β concentration in the group treated with BP250 mg/kg B.W. against to all other groups and there was a significant reduce ($p \leq 0.05$) in group administrated to BPA and their diet mixed with grape skin group compared with group of BPA only and significant increase compared with control groups.

The effect of exposing to (250 mg / kg B.W.) of BPA demonstrated a significant increase ($p \leq 0.05$) in serum of IL-2, IL-6 and TNF-α concentrations of female rats compared with all rest groups of the study. On the other hand, there was a significant differences ($p \leq 0.05$) in the group exposed to BPA and their diet mixed with grape skin group compared all other groups but it was insignificant ($p \geq 0.05$) changes between corn oil and normal saline groups. Lastly, from the results of the current study, we definite that there were protective role in the means of serum immunological parameters (C_3, IgG, IL-1β, IL-2, IL-6 and TNF-α) in BPA and their diet mixed with grape skin groups.

Table (4-3) the effect of BPA and grape skin on some serum immunological parameters in mature female rats (Means ± SE)

Parameters / Groups	C_3 mg/dl	IgG mg/dl	IL-1β pg/ml	IL-2 pg/ml	IL-6 pg/ml	TNF-α pg/ml
Normal saline group General control (GC) group (0.5ml/kg/B.W.)	C 133.91±9.28	C 1214.12±95.96	C 16.37±1.28	C 37.00±1.73	C 36.37±0.94	C 38.50±1.41
Corn oil group Vehicle control (VC) group (0.5ml/kg/B.W.)	C 131.88±9.96	C 1202.37±110.61	C 17.25±1.68	C 38.25±2.05	C 37.50±1.01	C 38.75±2.13
Grape skin group Treatment 1 (T1) group (5%mixed with food)	C 109.30±3.90	C 1000.25±52.74	C 10.37±0.94	D 29.37±0.94	D 28.75±1.03	D 28.25±1.26
Bisphenol-A- group Treatment 2 (T2) group (250 mg/kg/B.W.)	A 229.62±4.25	A 3143.00±185.73	A 226.25±3.01	A 231.62±2.87	A 267.00±1.73	A 268.87±2.78
Bisphenol-A-and grape skin group Treatment 3 (T3) group (250mg/kg/B.W of BPA +5% grape skin mixed with food)	B 171.18±15.50	B 1677.25±153.30	B 75.50±11.08	B 80.37±1.28	B 82.87±1.78	B 82.25±1.81

N=8

Different letters represent a significant difference at (p≤0. 05)

4-4- Effect of BPA and grape skin on some serum hormones levels in mature female rats

Data in table (4-4) explored presence of significant (P≤0.05) increase in the mean of LH serum level of female rat which was exposed to BPA (250 mg/Kg B.W) compared with all other groups. However, the use of grape skin with BPA caused significant decline (P≤0.05) in value of LH compared with the group that received grape skin only and control groups but still height than T2 group. On the other hand, there were no significant variations (P≥0.05) in concentration of LH in grape skin group and control groups.

Table (4-4) also showed there were significant increase ($p \leq 0.05$) in FSH serum level in the group administrated BPA (250 mg/Kg B.W) in comparison with other treated groups. While, there were non-significant changes (P≥0.05) in the value of FSH in the group exposed to BPA and their diet mixed with grape skin compared with grape skin group only and control groups.

Estradiol concentration was significantly (P≤0.05) increased in serum of mature female drenched to BPA (250 mg/Kg B.W) compared with all other four groups. On the other hand, the statistical analysis of variance found out significant (P≤0.05) decrease in the mean of estradiol concentration in female rats exposed to BPA and their diet mixed with grape skin in type progress compared with group give grape skin only and control groups. However, the results appeared non-significant changes (P≥0.05) in level of estradiol serum in grape skin group and control groups.

Table (4-4) the effect of BPA and grape skin on some serum hormones levels in mature female rats (Means ± SE)

Parameters / Groups	LH µIU/ml	FSH µIU/ml	Estradiol (pg/ml)
Normal saline group General control 1(GC) group (0.5ml/kg/B.W.)	CD 3.56±0.11	B 4.93±0.21	C 54.93±1.21
Corn oil group Vehicle control 2 (VC) group (0.5ml/kg/B.W.)	D 3.15±O.18	B 4.69±0.16	C 54.99±1.16
Grape skin group Treatment 1 (T1) group (5%mixed with food)	C 3.78±0.20	B 4.85±0.24	C 53.14±0.97
Bisphenol-A- group Treatment 2 (T2) group (250 mg/kg/B.W.)	A 6.56±0.20	A 7.40±0.11	A 73.49±1.40
Bisphenol-A-and grape skin group Treatment 3 (T3) group (250 mg/kg/B.W of BPA +5% grape skin mixed with food)	B 5.04±0.21	B 4.89±0.23	B 59.81±0.82

N=8

Different letters represent a significant difference at (p≤0. 05)

4-5- Effect of BPA and grape skin on histopathological changes in liver, kidney, ovary, uterus and spleen

4-5-1- The liver

The liver tissue examination of the control groups (normal saline and corn oil groups) has shown normal histological structure, normal central vein and hepatocytes arranged in irradiation manner (Fig 4-1 and 4-2) in treatment I group (grape skin group) also shown normal histological structure of central vein and surrounding hepatocytes (fig 4-3). On the other hand, liver sections of the female rats exposed to dose of 250 mg/kg B.W. of BPA have revealed dilated central vein, enlarged sinusoid space and pyknotic nuclei (Figure 4-4). While, results of microscopic examination of liver in female rats exposed to 250 mg/kg/B.W. of BPA and their diet mixed with grape skin showing histological changes with less degree in compare with group of BPA alone (Figure 4-5).

4-5-2-The kidney

In the present study, the results of tissue examination of kidney in normal saline, corn oil, and grape skin groups showed normal glomerulus and tubules (figures 4-6, 4-7 and 4-8). On the other hand, female rats exposed to 250 mg/kg B.W of BPA lead to histological changes kidney include massive area of necrosis in the tubular epithelial cells and atrophy in glumolar tuft (figure 4-9). Kidney tissue in female rats exposed to 250 mg/kg/B.W. of BPA and their diet mixed with grape skin showing moderate necrosis compared with that shown in BPA group (figure 4-10).

4-5-3- The ovary

The microscopic finding of ovaries in normal saline, corn oil and grape skin groups shows normal follicle growth (figures 4-11, 4-12 and 4-13). On

the other hand, histological changes in ovary of female rats exposed to 250mg/kg/B.W of BPA include presence of cyst-like structure (figure 4-14). Ovary tissue of female rats exposed to 250 mg/kg/B.W. of BPA and their diet mixed with grape skin showed presence of follicles in different stages of development (figure 4-15).

4-5-4- The uterus

Microscopic examination of uterus in female rats treated with normal saline, corn oil and grape skin groups showed normal distribution of uterine glands in the endometria with normal epithelial height (figs. 4-16, 4-17 and 4-18). While the uterus of rats exposed to 250mg/kg B.W of BPA revealed histopathological changes including increase in uterine luminal epithelial height and increase in number of uterine glands in the endometrium (fig 4-19).Tissue section in uterus of female rats exposed to 250 mg/kg/B.W. of BPA and their diet mixed with grape skin showed increase in uterine luminal epithelial height with less degree and increase number of uterine gland in the endometrium with less degree (fig 4-20).

4-5-5-The spleen

Microscopic examination of spleen in normal saline, corn oil and grape skin groups in female rats showed normal architecture of spleen and normal structure of white pulp and red pulp (figs. 4-21, 4-22 and 4-23).While the spleen of female rats exposed to 250mg/kg B.W of BPA revealed histopathological changes including massive necrosis of white pulp, which lead to disappear of multifocal white pulp (Figure 4-24**)** and female rats exposed to 250 mg/kg/B.W. of BPA and their diet mixed with grape skin showed moderate area necrosis of white pulp with presence of intact area of white pulp in spleen tissue (Figure 4-25).

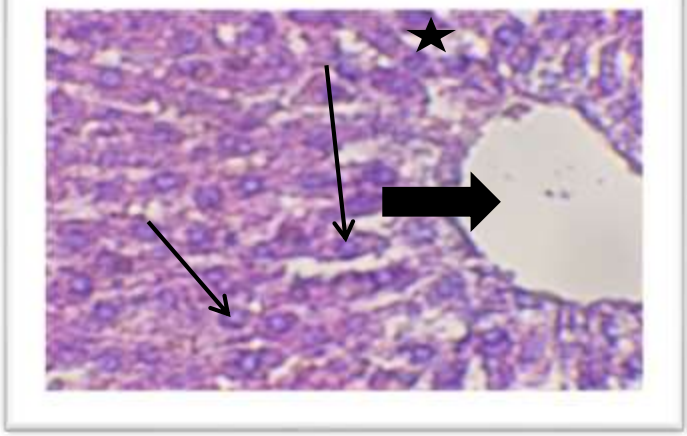

Figure 4-1: Light micrograph shows the histological structure of liver of normal saline group in rat shows normal central vein (thick arrow) hepatocytes (thin arrow) arranged in irradiation manner and small sinusoid space (stars). H&E,

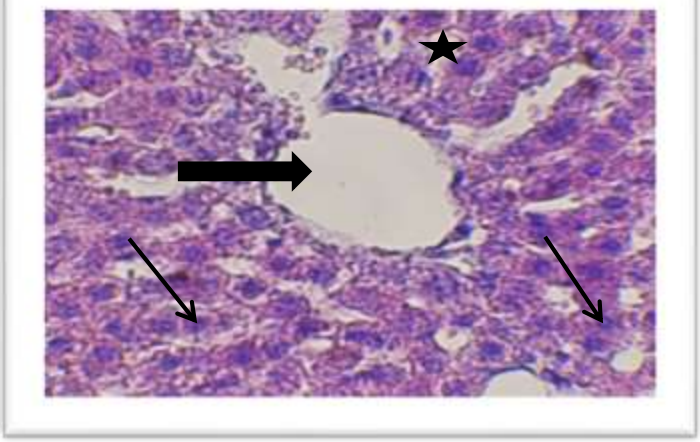

Figure 4-2: Light micrograph shows the histological structure of liver of corn oil group in rat shows normal central vein (thick arrow) hepatocytes (thin arrow) arranged in irradiation manner and small sinusoid space (stars) . H&E, 400x.

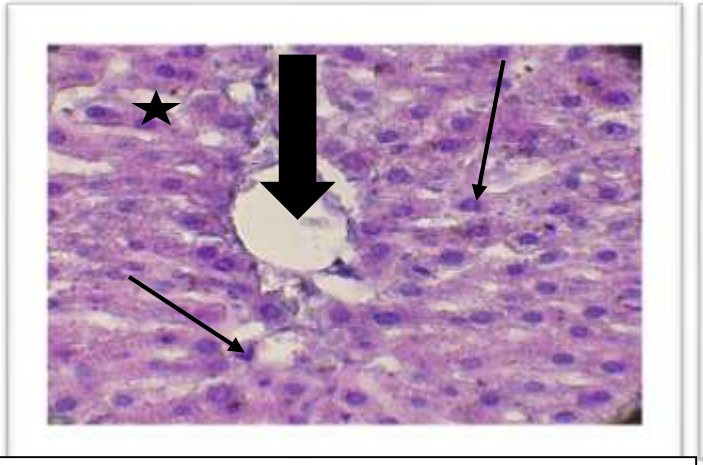

Figure 4-3: Photomicrograph of a liver section of rats fed with grape skin showing normal histological structure of central vein (thick arrow) surrounding hepatocytes **(**thin arrow**)** and small sinusoid space (stars) . H & E. 400x

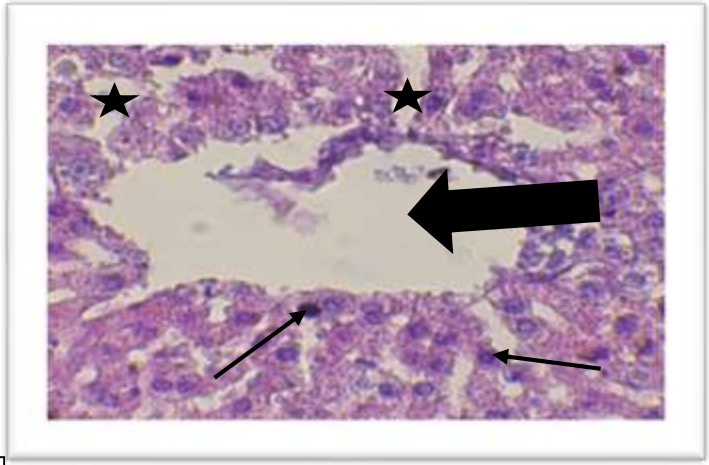

Figure 4-4: Light micrograph of histological changes in liver of female rat exposed to 250mg/kg B.W. of BPA shows delated central vein (thick arrow), enlarged sinusoid space (stars) and pyknotic nuclei (thin arrow) H&E.400x.

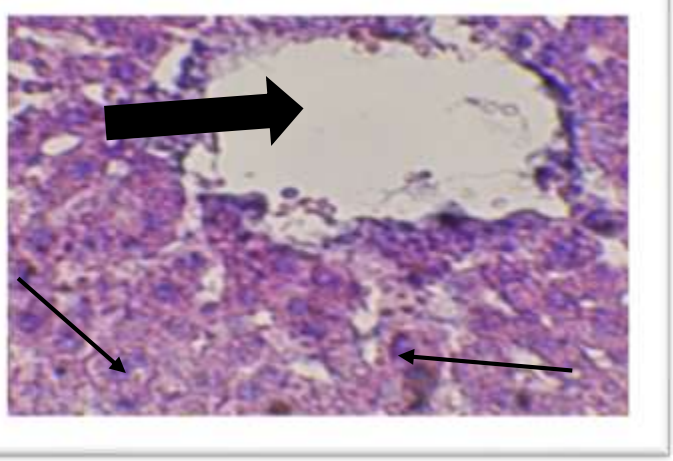

Figure 4-5: Photomicrograph of a liver section of rats exposed to 250 mg/kg/BW of BPA and their diet mixed with grape skin showing less delated central vein (thick arrow) and pyknotic nuclei with less degree (thin arrow) H&E.400x.

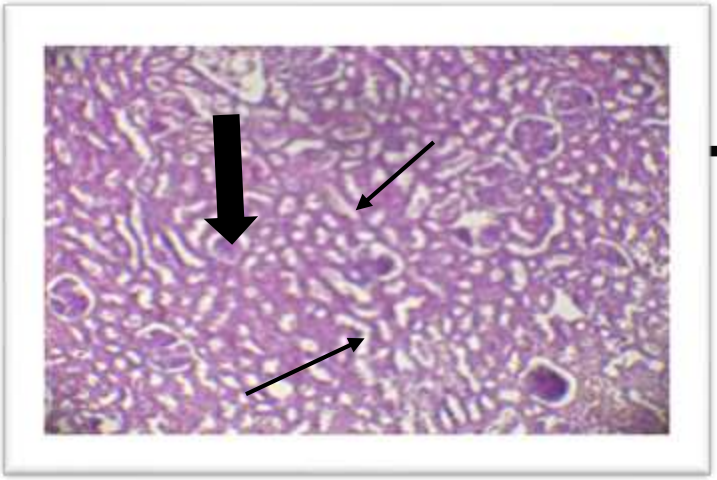

Figure 4-6: Photomicrograph of kidney tissue of normal saline group in rat showing normal glomerulus (thick arrows) and tubules (thin arrows).H & E. 100x.

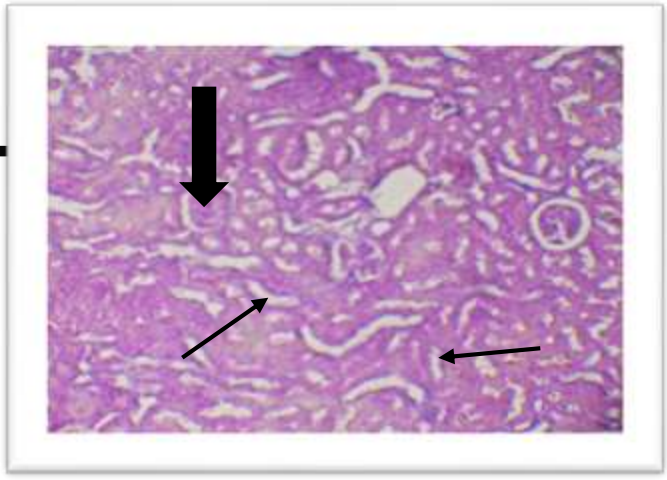

Figure 4-7: corn oil group section of kidney showing normal glomerulus (thick arrows) and tubules (thin arrows) H & E.100x.

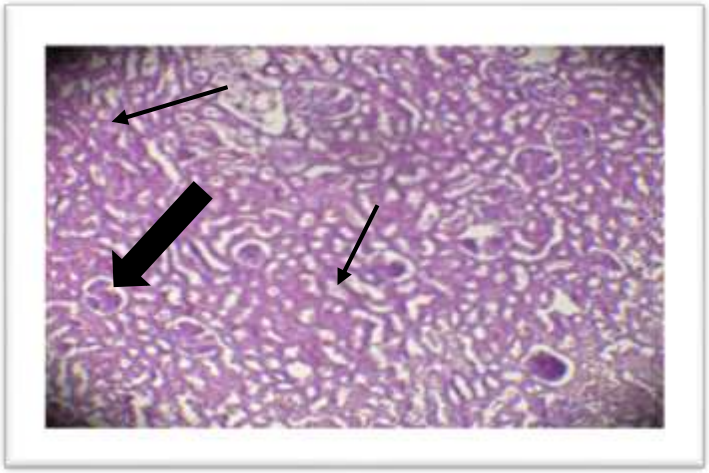

Figure 4-8: Photomicrograph of kidney tissue of rats fed with grape skin showing normal glomerulus (thick arrows) and tubules (thin arrows).H & E. 100x.

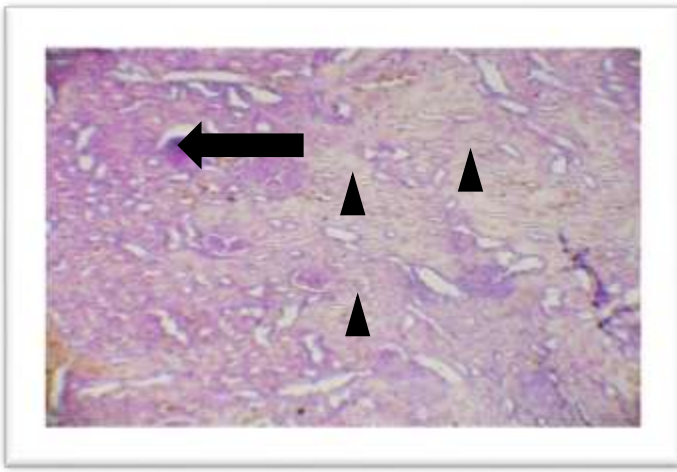

Figure 4-9: Light micrograph of histological changes in kidney of female rat exposed to 250mg/kg B.W. of BPA shows massive area necrosis(arrow heads) of the tubular epithelial cells and atrophy in glumolar tuft (thick arrow) .H & E. 100x.

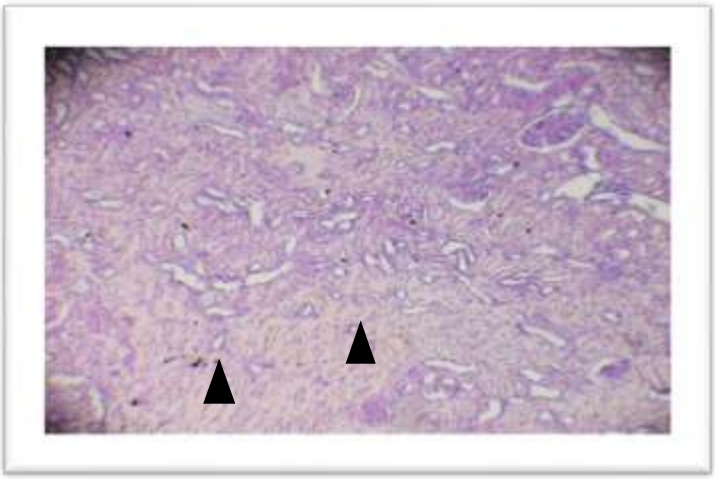

Figure 4-10: Photomicrograph of kidny section of rat exposed to 250 mg/kg/BW of BPA and their diet mixed with grape skin showing moderate necrosis (arrow heads) H & E. 100x.

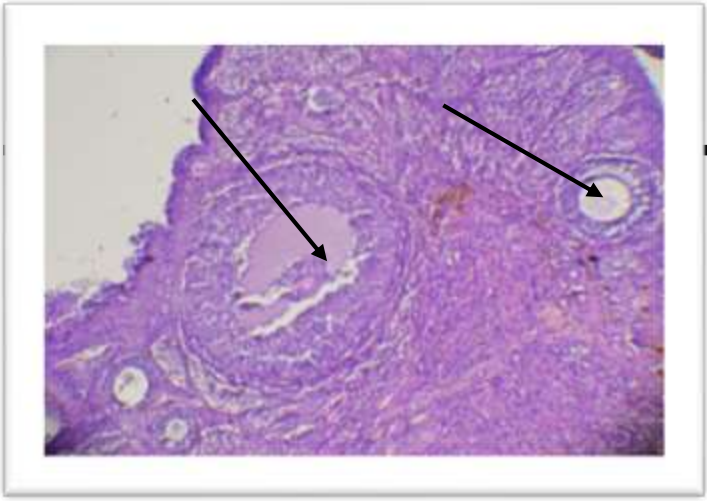

Figure 4-11: Light micrograph of ovary of normal saline group in rat shows follicles in different developmental stages (thin arrows).H&S, 100x.

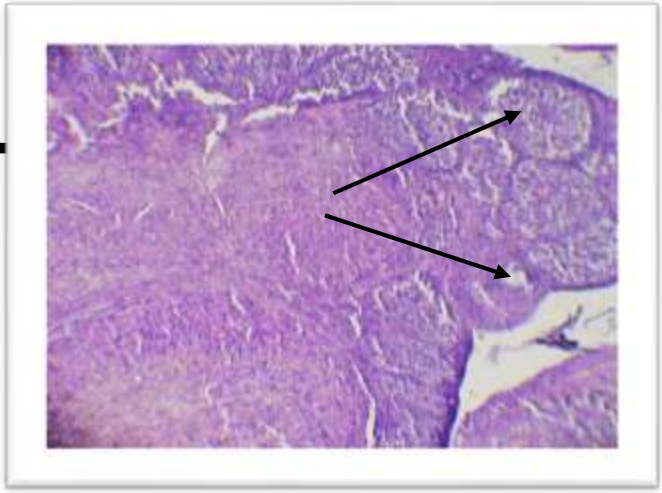

Figure 4-12: Light micrograph of ovary of corn oil group in rat shows normal follicle growth (thin arrows) H&S, 100x.

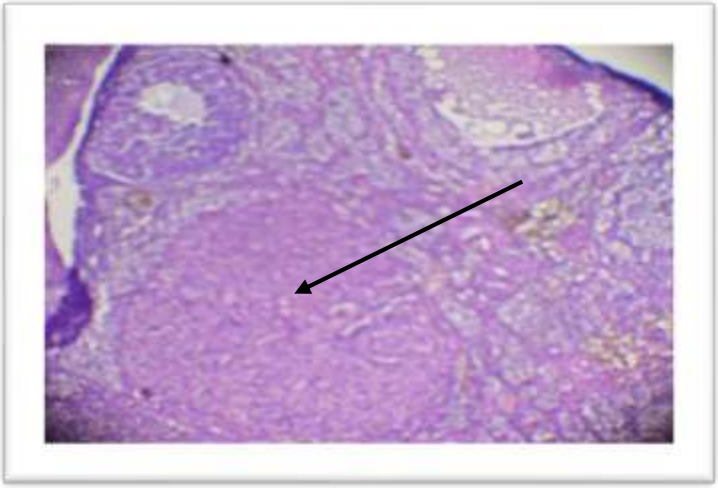

Figure 4-13: Light micrograph of ovary of rats fed with grape skin shows normal follicle growth (thin arrow) H&S,100x.

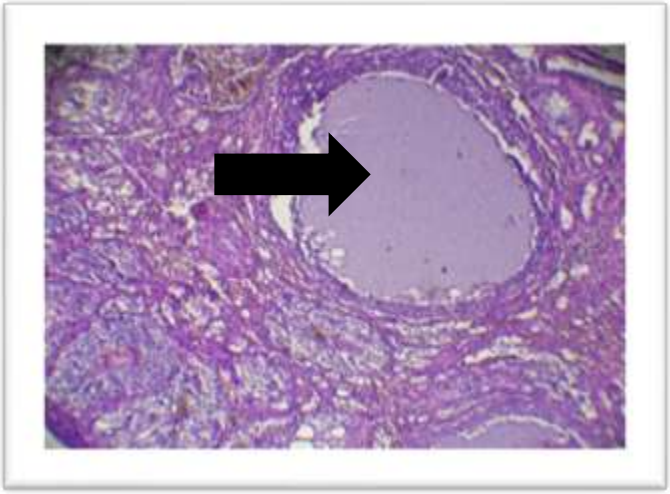

Figure 4-14: Light micrograph of rats ovary exposed to BPA 250mg/kg B. W. shows presence cyst-like structure (thick arrow).H&E,100 x.

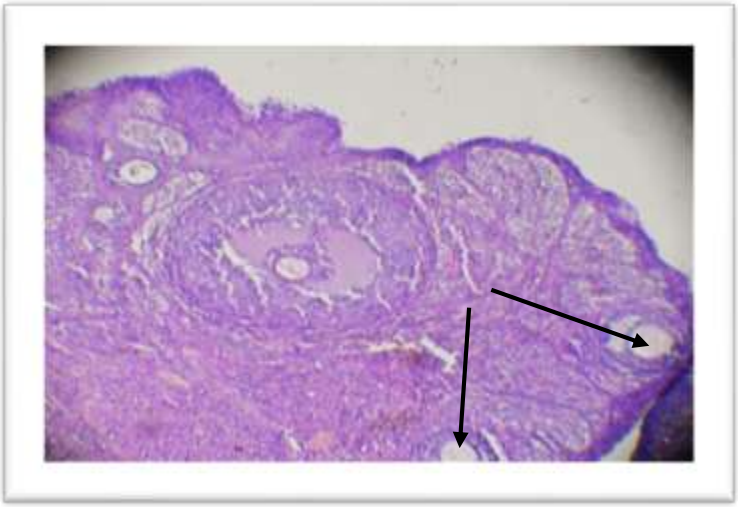

Figure 4-15: Photomicrograph of ovary section of rat exposed to 250 mg/kg/BW of BPA and their diet mixed with grape skin showing presence of follicles in different stages of development (thin arrows) . H&E,100 x.

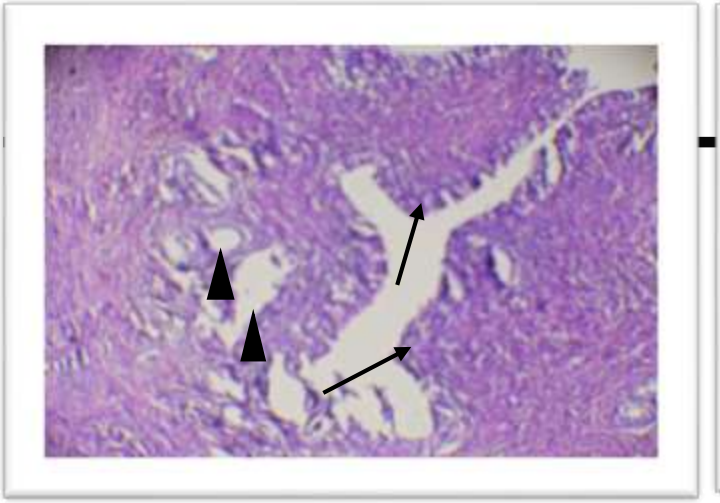

Figure 4-16: Light micrograph of uterus of normale saline group in rat shows normal distribution of uterine glands (arrow heads) in the endometria with normal epithelial height (thin arrows) H&E, 100x.

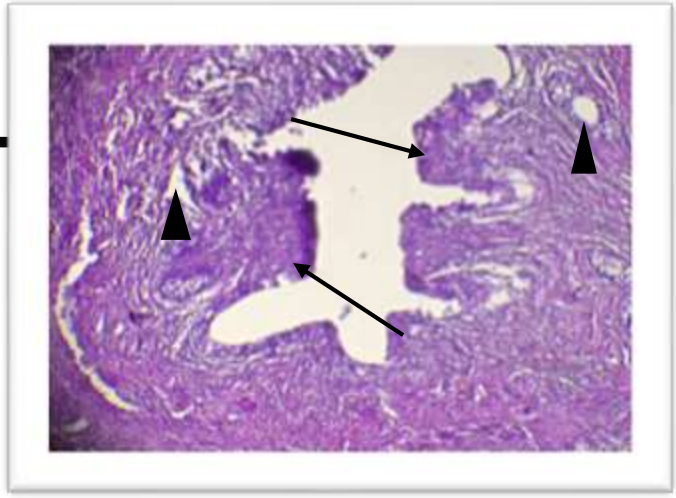

Figure 4-17: Light micrograph of uterus of corn oil group in rat shows normal distribution of uterine glands (arrow heads) in the endometria with normal epithelial height (thin arrows) H&E, 100x.

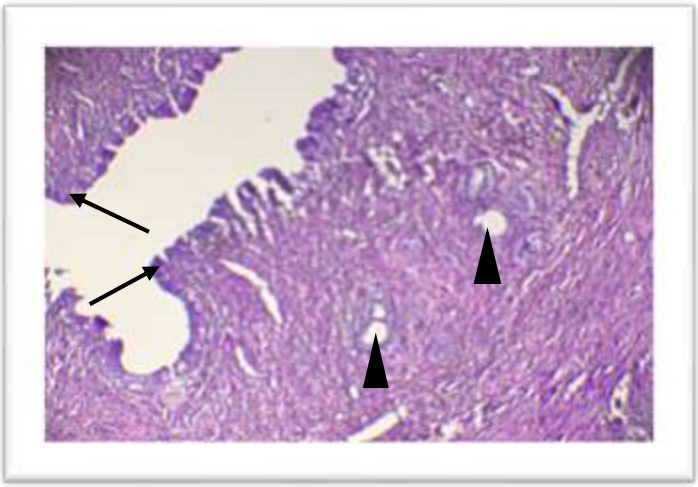

Figure 4-18: Light micrograph of uterus of rats fed with grape skin shows normal distribution of uterine glands (arrow heads) in the endometria with normal epithelial height (thin arrows) H&E, 100x.

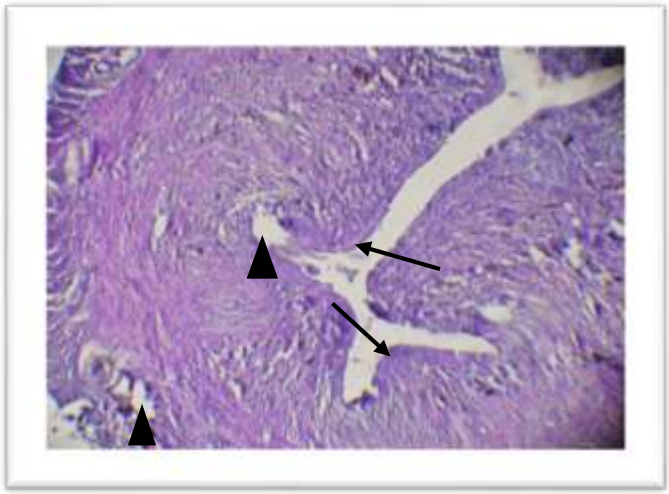

Figure 4-19 : Light micrograph of histological changes in uterus of female rats exposed to 250 mg/kg B.W. of BPA shows increase in uterine luminal epithelial height (thin arrow) increase number of uterine gland in the endometrium (arrow head). H&E, 100x.

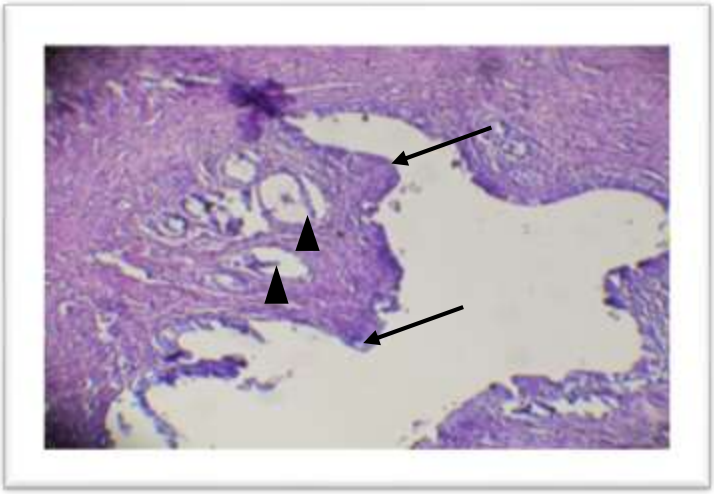

Figure 4-20: Photomicrograph of uterus section of rat exposed to 250 mg/kg/BW of BPA and their feed mixed with grape skin showing increase in uterine luminal epithelial height with less degree (thin arrow) increase number of uterine gland in the endometrium with less degree (arrow head). H&E, 100x.

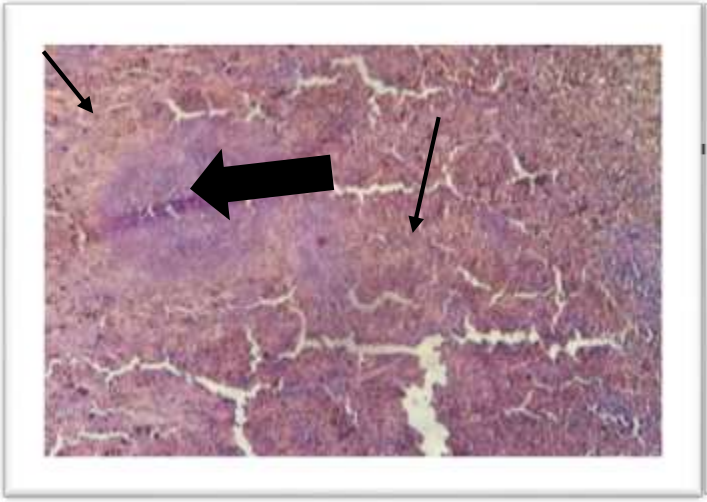

Figure 4-21: Light micrograph of spleen of normale saline group in rat shows normal archeticure of spleen and normal structure of white pulp(thick arrow) and red pulp (thin arrow) H&E, 100x.

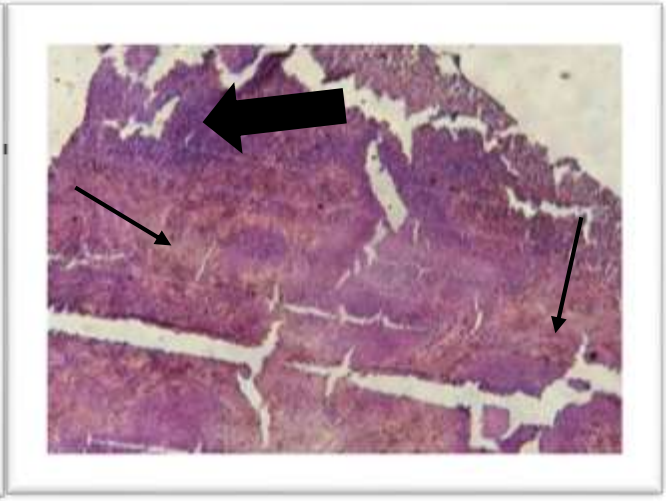

Figure 4-22: Light micrograph of spleen of corn oil group in rat shows normal archeticure of spleen and normal structure of white pulp(thick arrow) and red pulp (thin arrow) H&E, 100x.

Figure 4-21: Light micrograph of spleen of normale saline group in rat shows normal archeticure of spleen and normal structure of white pulp(thick arrow) and red pulp (thin arrow) H&E, 100x.

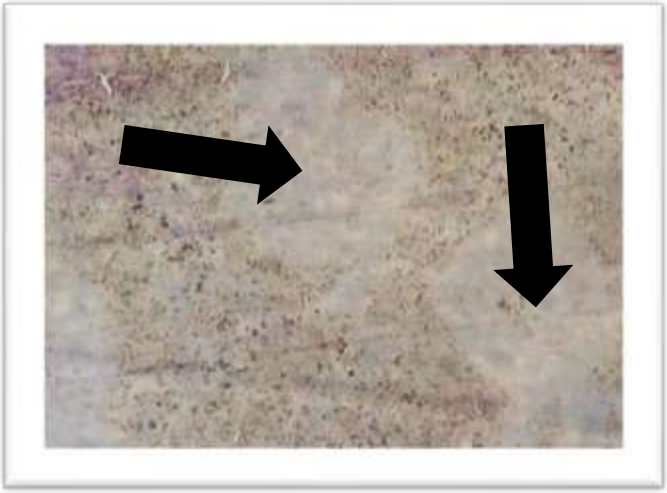

Figure 4-24: Light micrograph of histological changes in spleen of female rats exposed to 250 mg/kg B.W. of BPA shows massive necrosis of white pulp (thick arrow) which lead to disappear of multifocal white pulp H&E, 100x.

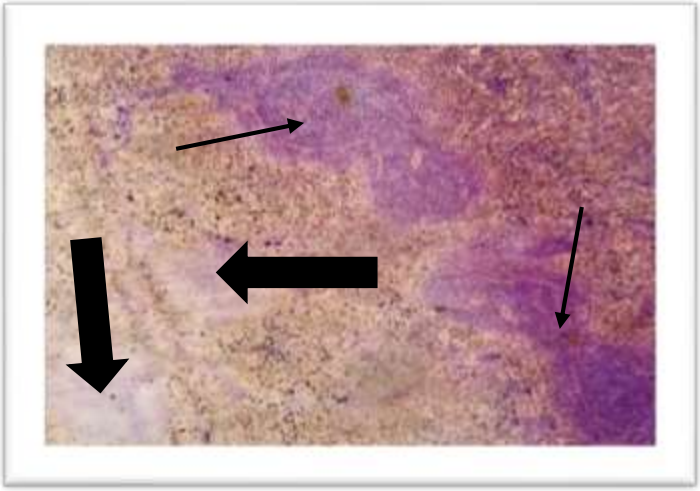

Figure 4-25: Light micrograph of spleen section of rat exposed to 250 mg/kg/BW of BPA and their diet mixed with grape skin showing moderate area necrosis of white pulp (thick arrow) with presence of intact area (thin arrow)H&E, 100x.

CHAPTER FIVE

DISCUSSION

5-Discussion

5-1-Hematological Parameters

The present results revealed significant increase in WBCs count of female rats which were exposed to 250 mg/kg/BW of BPA that appear in table (4-1-1) compared with other treated groups and these results were in coordinated with previous studies (Al-Mossawi, 2013). Other studies also showed increase in total number of leukocyte in rat exposed to 250 mg/kg/B.W. of BPA (Nitschke *et al*.,1988). The biology of immune cells is affected by BPA and its may play important role in the beginning or exacerbation of inflammatory conditions (Yang *et al*., 2009; Rogers *et al.*, 2013 and Ronna *et al.*, 2013).

In the present study, there is increases in WBCs value after the exposure to 250 mg/kg/B.W. of BPA, which may be explained by the role of BPA in induction of inflammatory conditions or may be due to increase percentage of neutrophil that appear in table (4-1-1). In addition to the above, the increasing number of WBCs could be due to stress that induced by BPA and stimulation of immune system. This result is in coordinate with Adedapo and Aiyelotano (2001).

While statistical, analysis in the present study revealed that there were significant decrease in WBCs count in group of BPA and their diet mixed with grape skin. Al-Jeboory *et al*., (2012) state that the use of proanthocyanidin caused significant decrease in number of WBC. This indicated that proanthocyanidin caused healing of inflammation and decreases the effect of BPA. Resveratrol is one of major constituents in grape skin, which lead to inhibition synthesis of eicosanoids in rat leukocytes (Kimura *et al*., 1985). Rotondo *et al.*, (1998) reported that resveratrol appear a strong inhibitory

influence on reactive oxygen species formed via human polymorphonuclear leukocytes.

The current results appeared that there is significant reduction in percentage of neutrophils in group of BPA and their diet mixed with grape skin. This result indicated reduction of inflammation and infiltration of neutrophil. As well as, there is non significant decline in percentage of eosinophil, basophil, which indicated reduction of stress. This result is reported also by (Al-Joobory *et al*., 2012). On the other hand, resveratrol is the main component of grape skin and has antileukemic activity in both *in vitro* and *in vivo* as reported by Gao *et al.,* (2002).

The current results showed that there was a significant decrease in the RBCs count and Hb level of female animals in response to exposure to 250mg/kg/BW of BPA that appeared in table (4-1-2) for females exposed to BPA compared with all other groups and these results were in coordinate with previous studies (Ulutas *et al*., 2011 and Yamasaki and Okuda, 2012).

Bisphenol A has similarity with estrogen effects resemble that in diethylstilbestrol so, the decrease of RBC count and Hb concentration in the present study may be resulted by decrease erythropoietin production due to estrogenic activity of BPA or its may cause by an increase in RBCs destruction (Godowicz,1990). Thomas *et al.,* (1982) reported a decrease in RBCs count and Hb concentration in rats exposed to BPA at high doses. This belong to number of factors, including a decrease in concentration of iron ion in the blood or shorter half life for red blood cells and their degradation as a result to changing in cell membrane permeability that make red blood cells more fragile and break more easier. Recently, the estrogens and diethylstilbestrol have ability to decrease erythropoietin production in rats

(Horiguchi *et al*., 2013).Whereas, RBCs count and Hb levels were increased significantly in group treated with BPA and their diet mixed with grape skin and these results were matched with previous studies.

Al-Razaq (2015) reported that the use of proanthocyanidin extracted from grape (one of the important compounds of grape skin and seed) causes a significant increase in RBCs counts and Hb level. This result may come from the antioxidant and oxygen free radical scavenger effect of proanthocyanidin, leading to improvement of RBCs counts. It may be also due to the ability of proanthocyanidin to improve kidney function such as secretion sufficient levels of erythropoietin hormone which in turn, affects the bone marrow to produce more red blood cells. This result is mentioned by Al-Jeboory *et al*., (2012) who indicated that proanthocyanidin cause increased in RBCs count. Finally, statistical analysis offered there were enhance role for grape skin against harmful effects for BPA in RBCs count, Hb level, WBCs count and differential WBCs count.

5-2-Biochemical Parameters

The current study showed that female animals treated with (250 mg/kg B.W) of BPA lead to significant increase in AST and ALT levels compared with all another groups (table 4-2). These results were matched with results obtained by Mourad and Khadrawy (2012) and Sangai and Verma (2012). Hassan *et al.*, (2012) were orally drenched BPA (0.1, 1, 10, 50 mg/kg/day) in rats for four weeks and reported that the dose of BPA (50mg/kg) lead to increase levels of ALT. Morever, such increase in serum AST and ALT was previously reported considering the toxicity of BPA that was resulted from 5 mg /kg/day of BPA in rats but not less than that dose (Korkmaz *et al*., 2010).

Moon *et al.*, (2012) and Mourad& Khadrawy (2012) were reported that exposure to BPA lead to changes in liver as a result increase oxidative stress that considered as indicator to increased levels of AST and ALT. Bindhumol *et al.,* (2003) and Kabuto *et al*., (2004) reported that BPA caused tissue injury in the kidneys, liver and other organs which leading to production of reactive oxygen species (ROS).

Hanioka *et al*., (2000) demonstrated that BPA accumulate in many tissues for example liver after BPA treatments to female rats. Enzymes of AST and ALT are considered reliable markers of hepatocellular injury or necrosis. Levels of these enzymes are raised in a variety of hepatic disorders. Enzymes of AST and ALT are released into the blood, When the liver hepatocytes are damaged which lead to significant increase in AST and ALT levels that indicates the damage to the cytosol and also to mitochondria. The significant dose-dependent change in the levels of ALT, AST showed the toxic effect of BPA. Therefore, it could be showed that the oxidative stress induced by exposure to the high dose of BPA (250 mg/kg/day) may reflect the disturbance in hepatic function (Mathuria and Verma, 2008). Which is appeared by significant increase in AST and ALT and confirmed by the presence of histopathological findings (figure 4-4) in the present study.

The reduction of BPA effect on hepatic function in group exposed to 250 mg/kg/day of BPA and their diet mixed with grape skin compared with BPA dose alone. Resveratrol is one of major constituents in grape skin when added to the diet of rats, lead to reduced level of AST and ALT in the serum and these result in coordinate with Hamadi *et al*., (2012).

Resveratrol caused significant reduce in levels of ALT and/or AST in animal models (mice and rats) which induced by different factors causing hepatotoxicity or hepatitis (lipopolysaccharide, naphthalene, CCL4 and

acetaminophen) (Sehirli *et al.*, 2008 and Farghali *et al.*, 2009). Administration of grape skin lead to enhancement of hepatic injury, which showed in the histological section of liver that appeared normal central vein, normal architecture and normal hepatocyte (figure 4-5).The hepatoprotective activity of proanthocyanidin included in grape skin may be due to the antioxidant and free radical scavenging features or enhanced antioxidant capacity of the liver (Cetin *et al*., 2008).

On the other hand, there is significant increase in blood glucose concentration in rats exposed to 250 mg/kg/day of BPA (table 4-2).These results matched with Alonso-Magdalena *et al*., (2010) who showed that the BPA disrupt glucose homeostasis in pregnant mice. In the current study, BPA at high dose lead to significant increase in blood glucose concentration in female rats and these results confirmed by some studies which showed that BPA causes hyperglycemia (D‟Cruz *et al*.,2012a and b and Sabanayagam *et al*.,2013).Bisphenol A affects the glucose metabolism by different mechanisms such as oxidative stress, inflammation, insulin resistance and β cell dysfunction. BPA has also been shown to cause, hyperinsulinemia and is considered a potential diabetogenic agent (Adachi *et al*., 2005; Alonso-Magdalena *et al*., 2006, 2010 and Jayashree *et al*., 2013).

In pancreatic islets, BPA may mimic the action of estradiol (E2) on blood glucose homeostasis through two different pathways: a rapid pathway via non-classical membrane estrogen receptor (Alonso-Magdalena *et al*., 2005) and a prolonged pathway via estrogen receptor (ER) (Takayanagi *et al*., 2006 and Ben-Jonathan *et al*., 2009).Over-stimulation of these receptors by BPA on the Beta cells increases insulin biosynthesis and secretion causing hyperinsulinemia that may precede insulin resistance (Jayashree *et al*., 2013). Hence, in the present study, it is possible that the estrogenic property of BPA

could have induced a state of insulin resistance resulting in simultaneous occurrence of hyperglycemia. There were improvement result in the mean of glucose serum in grape skin only and BPA and their diet mixed with grape skin group. These results come in coordinate with with previous studies (Nuttall *et al*., 2000; Bizeau and Pagliassotti, 2005 and Haring and Harris, 2011).

Many studies reported that resveratrol which is one of important constituents in grape skin when added to diet of rats exposed to BPA lead to reduction in glucose level compared with rats the animals exposed to BPA only and these studies showed there is strong relationship between hyperglycemia and oxidative stress (Miatello *et al*., 2005; Deng *et al*., 2008 and Bagul *et al.*, 2012). Some studies showed that resveratrol lead to decrease inflammation and oxidative stress in liver caused by hyperglycemia (Juan *et al*., 2002 and Hamadi *et al.*, 2012). Adisakwattana *et al*., (2010) reported that Proanthocyanidin is one of major constituents in grapes lead to significant decrease glucose concentration. Proanthocyanidin caused inhibition of pancreatic α-amylase and intestinal α-glycosidase.

In the other hand, grape seeds extracts caused a decline of serum glucose concentrations at 28 days because plant extract has high concentrations of flavonoid which lead to reduction the renal glucose re-absorption by inhibition of sodium- glucose symporters locate in proximal renal tubule (Hajati *et al*., 2015). This result on line with Sapwarobol *et al*., (2012) who studied the effect of proanthocyanidin(100mg and 300mg) on postprandial plasma glucose in healthy participants. Finally, the above results revealed that there were enrichment character in means of serum biochemical parameters (ALT, AST and Glucose) in grape skin with BPA group.

5-3-Immunological parameters

The present results revealed significant increase in C_3 and IgG concentration in group treated with 250 mg/kg B.W of BPA compared to every all groups (table 4-3) and these results were matched with previous studies (Haas *et al.,* 1997;Yoshino *et al*., 2003 and Goto *et al.*,2007). Ohshima *et al*., (2007) showed that offspring mice from the BPA administrated mothers group, when challenged with ovalbumin (a food antigen), showed a higher antigen-specific T cell proliferation rate, also increase in levels of ovalbumin-specific IgG were had no accumulate regulatory T cells. In the same study reported that BPA effect on the development of oral tolerance and lead to the rise of food allergies. Exposure to BPA lead to increase in concentration of IgG (Alizadeh *et al*., 2006).Bisphenol A moderately increased IgG a representative of Th1 type antibody, was also augmented (Goto *et al*., 2007).

Yoshino *et al.,* (2003) reported that BPA lead to significant increased in immunoglobin IgG antibodies in mice that were immunized with hen egg lysozyme. The effect of exposure to 250 mg / kg B.W. of BPA demonstrated a significant increase in serum of IL-1β, IL-2, IL-6 and TNF-α concentrations of female rats compared with all other groups. These results were matched with previous studies (Jontell *et al*., 1995; Tian *et al*., 2003; Yoshino *et al.*, 2003 and Yoshino *et al*., 2004). Asai *et al*., (2001) reported that the pathway by which BPA rises IL-6 release remains to be determined. There is no evidence for gender differences in circulating IL-6 or TNF_α concentrations in humans.

Bisphenol A caused an increase of innate immune response by increasing production of cytokines such as IL-1β and tumor necrosis factor (TNF-α) in macrophages. On the other hand, BPA stimulate both T and B cells in adaptive responses by using immune cells isolated from BALB/c mice

(Yamashita *et al.,* 2002).Yoshino *et al*., (2004) state that prenatal exposure to BPA lead to augmentation both of Th1 and Th2 responses in adulthood, these authors used IL-2 as marker for Th1 response. Hong *et al.,* (2004) report that BPA enhanced NO production in lipopolysaccharide (LPS) induced macrophages *in vitro.* In addition, showed that tumor necrosis factor-α (TNF-α) produced by macrophages.

Some studies showed that BPA lead to modulation of macrophage cytokines, which cause an in increase T cell activities (Yamashita *et al.,* 2002). Inadera *et al.,* (2000) demonstrated that different cells such as smooth muscle cells, macrophages, fibroblast and vascular endothelial cells produce MCP-1 in response to stimuli from TNF-α, IL-1β and LPS. Heinrich *et al.*, (2003) found that BPA stimulate the release of two inflammatory cytokines, IL-6 and TNF_α.The speculated on the mechanisms by which BPA affects their release. Intimately IL-6 involved with inflammatory states, immune responses, hematopoiesis and host defense mechanism. Vogt (2010) reported that immune system of human is enhanced when consumed of grape juice for four weeks. About 85 persons contributed in the study. Several measures of immune function were assessed on each person and all showed enriched concentrations. This is an important study in that one cannot assume taking a supplement for a couple of times a week will be as healthy as routine and consistent consumption.

On the other hand, the present study appeared significant decrease in ranges of serum concentrations in (C_3, IgG, IL-1β, IL-2, IL-6 and TNF-α) in group exposed to BPA and their diet mixed with grape skin group. Omayma *et al.*, (2015) mentioned that resveratrol which is one of major components for grape skin lead to decrease in IgG concentration in rats after 2, 4 and 6 weeks. Genistein, quercetin, and resveratrol are Phytochemicals that present

in grapes, which have also been shown to decrease pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α production (Tsang *et al*., 2005).

Seymour *et al.,* (2009) found that the plasma level of TNF-α and IL-6 reduced in rat fed high. Fat diet mixed with an anthocyanin, which is major bioactive compounds of grape skin. Grape seed polyphenols are considered active in inhibiting TNF-α (Shanmugam *et al*., 2008).Sung *et al*., (2009) estimated the protective effect of resveratrol tetramer against inflammation induced by LPS and found that only Inducible NOS (iNOS) showed dose dependent reduction in expression and no change in the TNF-α and IL-6 expression. Resveratrol treated with microglia cells lead to decrease in TNF-α concentration (Bi *et al.,* 2005). Peterson (2010) reported that resveratrol, caused decrease in Inducible NOS (iNOS) and IL-6 expression. From results of the present study, we discuss that there were defensive role for grape skin powder in means of serum immunological parameters (C_3, IgG, IL-1β, IL-2, IL-6 and TNF-α) to limit from toxic effects of BPA.

5-4- Hormonal parameters

Results of the present study showed there were significant increase in FSH and LH serum level in group administrated BPA (250 mg/Kg B.W) compared with all other groups. These results came in matched with previous studies (Tohei *et al*., 2001 and AL–Farhaan, 2015). Some studies suggested that increase in levels of LH hormone in females is an indicator on infertility because its secretion interfere with hypothalamic pituitary gonadal axis therefore raise of LH level in blood stream indicator for decrease production sexual steroids from ovary as in the case of premature ovarian failure and conjugate increase in LH level with poly cystic ovarian symptom and then lead to decrease infertility (AL–Farhaan,2015) and supported by results of

histology of ovary in the present study. Tohei *et al*., (2001) showed that BPA lead to increase in level of plasma LH is probably due to a reduction in the negative feedback regulation by estrogen.

Obaid (2016) reported that administrated (DHEA) Dehydroepiandrosterone which has mechanism similar to BPA in female rats lead to significant increase in FSH level as a result increase in FSH receptors at early phases of folliculogenesis and thus it is benefit for follicle recruitment. Some studies reported that there are increase in FSH level which is matched with raise in estradiol concentration with the histological alterations in ovaries which showed increase the texture of ovary beside the cystic follicles (Obaid, 2016) which appear in our study with histology.

While there was non-significant changes ($P \geq 0.05$) in value of FSH and LH in group exposed to BPA and their diet mixed with grape skin in compared with group administrated BPA only. Inhibitory effect of resveratrol on steroidogenesis was previsoly studied *in vitro* in different cell types adrenocortical and Leydig cell (Supornsilchai *et al.*, 2005 and Svechnikov *et al*., 2009) respectively. Histological results of present study revealed presence cyst like structure in the ovary of rat exposed to BPA (figure 4-14).Resveratrol have ability to induce apoptosis and decrease rat theca-interstitial cell growth by inhibiting the mevalonate pathway. On the other hand it also cause decrease availability of substrates of isoprenylation [farnesyl-pyrophosphate (FPP) and geranylgeranyl-pyrophosphate (GGPP)] (Israel *et al*., 2012).

Results of present study showed that female rats exposed to250mg/kg/B.W of BPA as appear in table (4-4) lead to significant increase in E2 level compared with all other groups. These results came in matched with previous studies (El-Mesalamy, 2009; Fernández, *et al*., 2010 and Xi *et al*., 2011).Gonadal steroidogenesis and sex hormone production are changed

as a result of hormones expression alteration at the hypothalamus and pituitary (Xi *et al*., 2011). The present results revealed that there is increased E2 level resulted from interfering of BPA with E2 synthesis pathways as mentioned by Grasselli *et al*.,(2010) via increasing expression level of Cytochrome P450 19a (CYP19a) as recorded by Arase *et al.*, (2011). Increasing of serum E2 level in the present study explained by possible interference of BPA mechanisms of local regulatory circuits of hypothalamus and pituitary.

The possible mechanism by which BPA effects on the local regulatory circuits of hypothalamus and pituitary is interfering with one or both of the primary forms of the estrogen receptor (ERα and ERβ) through the hypothalamic-pituitary-gonadal (HPG) axis (Adewale *et al*., 2009). Bisphenol A effect on some reproductive organs of female such as ovary, uterus,Vagina (indicator to estrogenic action), egg shape and fertility rate (Cabaton *et al.*, 2011 and Nah *et al*., 2011). The studies showed that females that have BPA concentration over normal levels are less fertility compared with control groups (Caserta *et al.*, 2013).Moreover, observed BPA effect on E2 might due to the additional estrogen potency of BPA relative to the existing endogenous estrogen equivalents. On the other hand, resveratrol is known to competitor with 17b-estradiol for estrogen receptors *in vitro* (Gehm *et al.*, 1997). Finally, present study presented meliorative effect in mean of estradiol concentration in group exposed of grape skin only and BPA and their diet mixed with grape skin group.

5-5- Histopathological changes in liver, kidney, ovary, uterus and spleen

5-5-1- Liver

In present study results of microscopic examination showed that there is effect of BPA on liver at high doses caused histopathological changes these results matched with previous results (Al Mossawi, 2013 and Al – Farhaan, 2015).Some studies appeared that histopathological changes in liver conjugated with increased ALT and AST in serum and appeared that BPA lead to rise of oxidative stress in liver ,also BPA increase infiltration of inflammatory cells and lead to different pathological changes in liver tissue in mouse therefore oxidative stress have harmful effect in hepatocytes (Bindhumal *et al*., 2003).

Moon *et al*., (2012) reported that BPA increase the damage in liver cells and defect in mitochondrial function because of ability of BPA to increase the oxidative stress. Results of many of studies appeared that BPA causes degenerative changes in hepatocytes (Boshra & Moustafa, 2011 and Roy *et al*., 2011). In present study it was noted that there is protective role for grape skin in recovery of hepatocytes compared with group exposed to BPA only and grape skin powder caused healing of hepatic injury induced by BPA, that was further confirmed by the histological section of liver which showed normal architecture, normal hepatocyte and normal central vein. Cetin *et al*., (2008) reported that the hepatoprotective activity of proanthocyanidin is one of the main components for grape skin and seed may be due to the free radical scavenging and antioxidant properties or enhanced antioxidant capacity of the liver.

5-5-2-Kidney

In the present study female rats exposed to high dose of BPA led to more deleterious histological changes in kidney represented with massive area necrosis of the tubular epithelial cells and atrophy in glomerular tuft this result agreement with (Al –Mossawi, 2013). Bisphenol A is an estrogenic endocrine disruptor molecule of phenolic structure used in plastics, which has renal elimination, and builds up when the glomerular filtration rate decreases .Phenols are uremic toxins has been associated with renal function impairment and vascular damage (González-Parra *et al.,* 2013). Bisphenol A is a molecule with structural similarity with phenols, which is a possible explanation for histological changes in kidney. The accumulation of metabolites of BPA and inability of kidney to excrete them might affect the kidney tissues of treated rats. On the other hand, the kidney's histological changes might be caused by increase formation of ROS and increase the oxidative stress induced by BPA (Asahi *et al*., 2010).

The previous histopathological changes was reported by Tyl *et al.*, (2008) and Manikkam *et al.,* (2013). The present study noted that grape skin has defensive effect against harmful changes in kidney tissue by causing healing of renal damage due to its antioxidant and free radical scavenging properties. In addition, it is able to attenuate the increase in urea (Wei *et al*., 2012). This is well confirmed by the histological section of kidney that showed decrease the area of necrosis. Ulusoy *et al*., (2012) reported that proanthocyanidin has protective effect against induced nephrotoxicity.

5-5-3-Ovary

Numerous studies suggest that the female ovary is sensitive to the endocrine disruptors (Uzumcu and Zachow, 2007; Badraoui *et al.,* 2010 and

Padmanabhan *et al.*, 2010). Results of microscopic examination in present study appeared different histopathological changes in female rats ovary administrated with BPA at high dose include different abnormal alterations such as presence of cystic-like structure. Whetherill *et al*., (2007) and Lee *et al.*, (2013) reported that mature female rats exposed to BPA for 90 day appeared significant histological changes in apoptosis of granulosa cells. Bisphenol A lead to decrease in oocytes in women (Bloom *et al*., 2011). Bisphenol A effect on FSH receptors in ovary and changes in granular oocytes therefore, BPA effect on regulation of transcription process and hormonal activity that play important role in replication of cells and occur essential conditions for growth of follicles (Simoni *et al*., 1997).

Histological finding of other study had shown necrosis, hemorrhage and presence of large number of ataractic follicles with no indication of ovulatory process in rats exposed to high dose of BPA that indicate the ovo-toxic nature of BPA (AL–Farhaan, 2015). Other study reported that natural and synthetic estrogenic compounds inhibitor for germ cell cyst breakdown and primordial follicle formation (Jefferson *et al.*, 2006). In addition, the evidence that BPA has functions same that of estradiol (Zhang *et al.*, 2012).These histological changes could account for some of the effects of BPA on female reproductive disorders, especially that concerning with ovary associated pathologies such as altered cyclicity and fecund ability, polycystic ovary syndrome, and precocious menopause (Crain *et al.*, 2008).In the present study results of microscopic examination in ovary tissue appeared that there are significant enhancement for grape skin in group treated with BPA and their diet mixed with grape skin that may be due to ameliorative effect of proanthocynidin that was previously reported by (Al-Razaq, 2015).

5-5-4-Uterus

The microscopic finding in present study of uterus of rats treated with high doses of BPA (250mg/kg B.W) revealed histopathological changes including increase in uterine luminal epithelial height and increase number of uterine gland in the endometrium. These results agreed with previous studies (AL Mossawi, 2013) that showed presence of histopathological effect of BPA on reproductive organs such as uterus. Some studies showed that BPA effect on uterus tissue by its estrogenic nature (Signorile *et al*., 2010).Therefor, BPA have ability to react with estrogen receptors and lead to appear disorders associated with estrogen (Gobellis *et al*., 2009).

Estrogen have uterotrophic effects such as hypertrophy of uterine epithelial cells (Branham *et al*., 1993; Cooke *et al*., 1997 and Katsuda *et al*., 1999) and stimulation of stromal and myometrial layers (cook *et al*., 1997 and Hunter *et al*., 1999). Previous study done on the ovariectomized mouse showed that both BPA and estrogen caused significant increase in the luminal epithelial cell height and thickness of uterus in both layers (stromal and myometrial) as reported by (Papaconstantinou *et al*., 2000). In the present study results of microscopic examination in uterine tissue appeared that there are significant enhancement for grape skin in group treated with BPA and their diet mixed with grape skin this result is in agreement with Khattab *et al*., (2010) who indicated that grape extract alleviate reproductive toxicity caused by aluminum chloride in male rats.

5-5-5-Spleen

In present study results of microscopic examination in spleen tissue showed that there is histopathological changes in female rats spleen administrated with 250 mg/kg/B.W of BPA include massive necrosis of white pulp, which lead to disappearance of multifocal white pulp these results

agreed with previous studies (Amaravathi *et al.*, 2012 and Hussein, 2015). Spleen tissue showed multiple focal necrosis and lymphocytic depletion in mice exposed to BPA for 4 weeks (Dawoud *et al.*, 2009). Bisphenol A lead to the migration of the neutrophils into peritoneal cavity but decreased their phagocytic activity, also BPA reduced population of macrophage and lymphocytic in the spleen and its accumulation in the infected foci (Sugita-Konishi *et al*., 2003).

Some studies showed that the histological changes of spleen of female rats received BPA such as congestion around blood vessels with fibrosis, atrophy in white pulp and edematous in red pulp (Hussein, 2015).In the present study results of microscopic examination in spleen tissue appeared that there is protective role for grape skin to decrease the toxicity of BPA. These results agreed with previous studies Karabulut *et al*., (2006) reported that there were ameliorative effects of resveratrol, which is one of main components in grape skin on spleen in rats subjected to ischemia reperfusion. There are many of symptoms that noted in some tissues such as necrosis, infiltration of inflammatory cells such as monocyte and lymphocyte that produces from effect of BPA on smooth endoplasmic reticulum that remove toxic effect of BPA as immunic reaction against disruptors that occur in organ exposed to BPA in the case of bleeding that occur in many of organs as a result rupture of blood vessels walls (Khan *et al*., 2002).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6-1-Conclusions

From the results of the present study, it was concluded that:

1-Bisphenol A leads to a negative results on hematological, biochemical, immunological, hormonal parameters in addition to histopathological changes in organs of liver, kidney, ovary, uterus and spleen.

2-Grape skin has protective role in some states to enhancement the harmful effects of BPA e.g. it causes significant decrease in concentrations of C3, IgG, IL-1β, IL-2, IL-6 and TNF-α .

3-Grape skin only induce no change in hematological, biochemical, immunological, hormonal parameters in addition to histopathological changes in organs of liver, kidney, ovary, uterus and spleen.

4-Corn oil as a vehicle does not have significant effects in studied parameters compared with normal saline group.

6-2-Recommendations

Due to the serious adverse effects of BPA and enhancement roles of grape skin, it is recommended that:

1- Epidemiological study should be done to monitor the level of BPA in Iraqi people and particularly in plastic manufacturing workers.

2- A further studies about effect of BPA on type 2 diabetes mellitus, polycystic ovary and obesity.

3-Pregnant females should avoid exposing to BPA because the perinatal period seems to be a critical" exposure window" for BPA to affect reproductive neural circuits in hypothalami of both females and males.

4-Regular monitoring to the level of BPA in baby bottles and different food cans especially that contains fatty products.

5-Study the effect of BPA on thyroid gland and thyroid hormones should be done.

6- Other studies are necessary to use different doses of grape skin on other toxic substances.

7-Extraction of the active substances in grape skin especially resveratrol and determination of biological activity of extract components.

CHAPTER SEVEN
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7-References

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الخلاصة

الدراسة الحالية أجريت في كلية العلوم بالتعاون مع كلية الطب البيطري /جامعة كربلاء لدراسة تأثير التعرض للبسفينول أ على بعض المعايير الدمية والكيموحيوية والمناعية والهرمونية أضافة الى التغيرات النسجية في الأعضاء المختلفة لاناث الجرذان البيضاء.

استمرت التجربة لمدة 30 يوم حيث قسمت أناث الجرذ البالغة (بعمر 14-16 أسبوع) (40 أنثى جرذ) الى خمس مجاميع (8 لكل مجموعة) (مجموعتي سيطرة وثلاث مجاميع معاملة). المجموعة الأولى اعتبرت (مجموعة سيطرة عامة) وجرعت الحيوانات فمويا 0.5 مل/كغم من وزن الجسم من المحلول الملحي يوميا, المجموعة الثانية جرعت فمويا بعالق زيت الذرة فقط وبجرعة 0.5 مل/كغم من وزن الجسم واعتبرت مجموعة (سيطرة للمادة الحاملة), المجموعة الثالثة اعتبرت (كمجموعة المعاملة الأولى) حيث مزج غذائها مع مسحوق جلد ثمرة العنب بنسبة 5% ولم تجرع , المجموعة الرابعة (مجموعة المعاملة الثانية) جرعت جرذانها فمويا بجرعة 250 ملغم /كغم من وزن الجسم من مادة البسفينول أ المعلق بمحلول زيت الذرة يوميا, المجموعة الخامسة اعتبرت (مجموعة المعاملة الثالثة) حيث جرعت جرذانها فمويا بجرعة 250 ملغم /كغم من وزن الجسم من مادة البسفينول أ المعلق بمحلول زيت الذرة يوميا وغذائها الممزوج مع مسحوق جلد ثمرة العنب بنسبة 5%.

نتائج الدراسة الحالية بينت أن مجموعة المعاملة الثانية سببت نقصان معنوي ($p\leq0.05$) في تركيز الهيموغلوبين وعدد كريات الدم الحمراء ولكن عدد كريات الدم البيضاء ونسبة خلايا الدم البيضاء العدلة ازداد معنويا ($p\leq0.05$) في اناث الجرذان المعاملة مع البسفينول أ مقارنة مع كل المجاميع الأخرى , بينما حيوانات مجموعة المعاملة الثالثة أظهرت تحسن معنوي في كل المعايير الدمية متمثلة بارتفاع معنوي ($p\leq0.05$) في عدد كريات الدم الحمراء ومستوى الهيموغلوبين بالإضافة الى انخفاض معنوي ($p\leq0.05$) في عدد كريات الدم البيضاء ونسبة خلايا الدم البيضاء العدلة بالمقارنة مع البسفينول أ لوحده لتصل مستوياتها الى معدلات مجموعتي السيطرة ومجموعة المعاملة الأولى في معظم المعايير الدمية المذكورة انفا.

نتائج المعايير الكيموحيوية بينت أن هناك زيادة معنوية ($p\leq0.05$) في مستويات نشاط أنزيمي AST و ALT بالإضافة الى مستوى السكر في مصل اناث الجرذان المعاملة مع البسفينول أ فقط الذي سبب تأثيرات ضارة على الكبد والكلية, بينما مجموعة المعاملة الثالثة أثبتت بأن هناك تحسن

معنوي ($p\leq0.05$)في معدل نشاط أنزيمي AST و ALT ومستوى السكر بالمقارنة مع مجموعة البسفينول أ فقط (مجموعة المعاملة الثانية) ليصل مستوى السكر لمعدلات مقاربة لمجموعتي السيطرة ومجموعة المعاملة الاولى بينما نشاط أنزيميAST و ALT لم تصل معدلاتها الى معدلات مجموعتي السيطرة. أشارت نتائج الجداول المناعية الى ان البسفينول أ أدى الى حصول ارتفاع معنوي ($p\leq0.05$) في تراكييز المتمم-3- والامينوكلوبيولين-ج- والانترلوكين-1بيتا- و انترلوكين-6- و انترلوكين-2- وعامل النخر الورمي- ألفا-مقارنة مع كل مجاميع الدراسة الاخرى بينما جلد ثمرة العنب مع البسفينول أ سبب اختزال في مستويات المصل في كل المعايير المناعية بالمقارنة مع مجموعة البسفينول أ فقط غير ان الانخفاض لم يصل الى مستوى مجموعتي السيطرة ومجموعة المعاملة الأولى. نتائج الهرمونات أظهرت بأن هناك زيادة معنوية ($p\leq0.05$) في مستويات هرمون الاستروجين والهرمون اللوتيني والهرمون المحفز لنمو الجريبات في مجموعة المعاملة الثانية، بينما انخفضت معدلات هذه الهرمونات معنويا ($p\leq0.05$) في مجموعة المعاملة الثالثة بالمقارنة مع مجموعة البسفينول أ لوحدها غير ان الانخفاض لم يصل الى مستوى مجموعتي السيطرة ومجموعة المعاملة الأولى. نتائج الفحص المجهري للمقاطع النسجية بينت وجود تغيرات نسجية في أعضاء الكبد والكلية و المبيض والرحم والطحال في مجموعة المعاملة الثانية متمثلة بوجود أنوية متغلظة في خلايا الكبد و تنخر في الخلايا الطلائية للنبيبات الكلوية في الكلية مع وجود تراكيب كيسية في المبيض إضافة الى ارتفاع في طول الخلايا الطلائية المبطنة للرحم و تنخر واسع في اللب الأبيض للطحال, بينما حيوانات المجموعة الثالثة أظهرت تحسن في شدة التغيرات النسجية في الأعضاء المدروسة مقارنة مع مجموعة البسفينول أ لوحدها. من نتائج الدراسة الحالية نستنتج بأن البسفينول-أ- يؤدي الى نتائج سلبية على المعايير الدمية والكيموحيوية والمناعية والهرمونية بالأضافة الى التغيرات المرضية النسجية في أعضاء الكبد والكلية والمبيض والرحم والطحال وجلد ثمرة العنب يمتلك دور وقائي في معظم الحالات لتحسين التأثيرات الضارة للبسفينول-أ- مثل أنه يسبب نقصان معنوي في تراكيز المتمم-3- والامينوكلوبيولين-ج- والانترلوكين-1بيتا- و انترلوكين-6- و انترلوكين-2- وعامل النخر الورمي- ألفا وجلد ثمرة العنب فقط لايحث تغير في المعايير الدمية والكيموحيوية والمناعية والهرمونية بالأضافة الى التغيرات المرضية النسجية في أعضاء الكبد والكلية والمبيض والرحم والطحال وزيت الذرة كمادة حاملة لايمتلك تأثيرات معنوية في المعايير المدروسة مقارنة مع مجموعة السيطرة العامة.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء/ كلية العلوم

قسم علوم الحياة

دراسة التغيرات الفسلجية والنسيجية والمناعية في أناث الجرذان المعرضة للبسفينول (أ) والدور الوقائي لجلد ثمرة العنب

رسالة مقدمة الى

مجلس كلية العلوم – جامعة كربلاء

كجزء من متطلبات نيل درجة الماجستير في علوم الحياة

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