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EVALUATION OF THE PROTECTIVE EFFECTS OF SILYMARIN AND TEMPOL AGAINST CISPLATIN INDUCED HEPATOTOXICITY

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I. DECLARATION FORM

I declare that: this thesis is my own work, I have revealed no unethical behaviours at all from its planning to its writing, I have provided all the information taking place in this thesis according to ethical academic rules, I have displayed references for all information and comments that have not been obtained as a result of this study and I have included all these references in the reference list, I have not performed any copyright infringement acts during the study period and its writing.

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III. CONTENTS

IV. ABBREVIATIONS and SYMBOLS LIST

ALT: Alanine Aminotransferase

AST: Aspartate Aminotranferase

CAT: Catalase

COX-2: Cyclooxygenase-2

ELISA: Enzyme-Linked ImmunoSorbent Assay

GIT: Gastrointestinal Track

GSH: Glutathione

H&E: Hematoxylin and Eosin

I.C: Inhibitory Concentration

I.P: Intra Peritoneal

I.V: Intra Venous

LD: Lethal Dose

LDL: Low Density Lipoprotein

MDA: Malondialdehyde

MPO: Myeloperoxidase

MRI: Magnetic Resonance Imaging

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NAPQI: N-acetyl-P-benzoquinone imine

NSAIDs: Non-Steroidal Anti-inflammatory Drugs

PSA: Prostate-Specific Antigen

ROS: Reactive Oxygen Species

SOD: Superoxide Dismutase

TAS: Total Antioxidant Status

TNF α: Tumour Necrosis Factor Alpha

TOS: Total Oxidant Status

TPA: 12-O-tetradecanoylphorbol

TPN: Total Parenteral Nutrition

UV: Ultra Violet

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1. ÖZET

Sıçanlarda Silymarin ve Tempolün Sisplatin Kaynaklı Hepatotoksisite Üzerine Etkilerinin İncelenmesi

Tayf Alqozbakr; **Danışman:** Prof.Dr. Fikret Vehbi İzzettin, Klinik Eczacılık Anabilim Dalı

Amaç: Çalışmamızda sıçanlarda silymarin ve tempolün sisplatin kaynaklı hepatotoksisite üzerine koruyucu etkilerinin incelenmesi amaçlanmaktadır.

Gereç ve yöntem: Sıçanlar her grupta altı hayvan olacak şekilde altı gruba ayrılmıştır. Üç kontrol grubunun her birine yedi gün boyunca serum fizyolojik (1 ml/kg; oral gavaj), tempol (15 mg/kg; i.p.) veya silymarin (100 mg/kg; oral gavaj) uygulanmıştır. Toksisite grubuna yedinci günde tek doz sisplatin uygulanmıştır. İki tedavi grubuna yedinci gün uygulanan sisplatin ile birlikte 7 gün süreyle tempol veya silymarin verilmiştir. Sisplatin uygulanmasından 24 saat sonra karaciğerleri çıkarılmıştır. Daha sonra malondialdehit (MDA), miyeloperoksidaz (MPO) ve toplam antioksidan durum (TAS) ölçülmüş ve de histopatolojik inceleme yapılmıştır.

Bulgular: Tedavi gruplarının MDA düzeyleri; tempol (58.46±5.34 U/g doku); ve silymarin (80.65±6.9 U/g doku) sisplatin toksisite grubuna göre (176.3±21.55) düşük bulunmuştur ($p<0.05$). Aynı zamanda, tedavi gruplarının MPO düzeyleri; tempol (23.19±0.7 U/g doku); ve silymarin (23.37±1.2 U/g doku) sisplatin toksisite grubuna göre (31.81±1.6 U/g doku) anlamlı derecede yüksek bulunmuştur ($p<0.05$). Tedavi gruplarındaki TAS düzeyleri; tempol (1.65±0.14 U/g doku) ve silymarin (1.67±0.19 U/g doku) Toksisite grubuna göre anlamlı derecede yüksek bulunmuştur (0.44±0.13 U/g doku) ($p<0.05$). Bu sonuçlar histopatolojik incelemelerle uyumlu bulunmuştur.

Sonuç: Sonuç olarak, bu çalışmada tempol ve silymarinin sisplatin kaynaklı hepatotoksisitenin önlenmesinde etkili olduğu gösterilmiştir.

Anahtar sözcükler: hepatotoksisite, sisplatin, antioksidan, karaciğer

2. SUMMARY

Evaluation of the Protective Effects of Silymarin and Tempol on Cisplatin-Induced Hepatotoxicity in Rats

Tayf Alqozbakr; **Supervisor**: Prof.Dr. Fikret Vehbi Izzettin; Clinical Pharmacy Department

Aim: The aim of our study is to evaluate the protective effects of silymarin and tempol against cisplatin induced hepatotoxicity in rats.

Material and Method: Rats were divided into six groups each consisting of six animals. Each the three control groups were adminstrated either saline or tempol (15 mg/kg i.p.) or silymarin (100 mg/kg p.o.) for seven days. Toxicity group of rats was given cisplatin (10 mg/kg i.p.) as a single dose on seventh day. Two treatment groups were either given tempol or silymarin for seven days with cisplatin on the seventh day. Livers were removed after 24 hours of cisplatin administration. Then Malondialdehyde (MDA), myeloperoxidase (MPO), and total antioxidants status (TAS) were measured and a histopathological examination was also done.

Results: MDA levels of treatment groups: tempol (58.46±5.34 U/g tissue); and silymarin (80.65±6.9 U/g tissue) were significantly low in comparison with cisplatin toxicity group (176.3±21.55) ($p<0.05$). Also, MPO level of treatment groups: tempol (23.19±0.7 U/g tissue); and silymarin (23.37±1.2 U/g tissue) were significantly low when compared with cisplatin toxicity group (31.81±1.6 U/g tissue) ($p<0.05$). TAS level in treatment groups: tempol (1.65±0.14 U/g tissue); silymarin (1.67±0.19 U/g tissue) were significantly high when compared with toxicity group (0.44±0.13 U/g tissue) ($p<0.05$). These results were coordinated with histopathological examination**.**

Conclusion: This study shows tempol and silymarin are effective in preventing cisplatin induced hepatotoxicity.

Key Words: hepatotoxicity, cisplatin, antioxidant, liver.

3. INTRODUCTION and AIM

Medicine induced hepatic toxicity is considered the main cause of acute liver disease. Generally, the impact of drug inducing hepatotoxicity is significant and only 20% of drug induced liver disease cases can be cured with supportive therapy. Sometimes just liver transplantation is curative; therefore, early diagnosis and supportive therapy are recommended (Ostapowciz, 2000). Traditionally, hepatotoxicity is the main post marketing reason for drug withdrawal (Andrale, 2005). Physicians who are concerned with hepatotoxicant drugs should weight the benefit and potential risks and they should follow a proper guideline to avoid hepatotoxicity (Chang, 2006).

The biotransformations of drugs that occur in liver include several steps; but the oxidation by cytochrome P450 enzymes is the most important one. After biotransformation, N-acetyl transferase and glutathione transferase enzymes will be conjugated with the metabolites and will result in eradication of free radicals. Genetic individual variations will result in polymorphism of these enzymes, this variation will lead to a discrepancy in drugs metabolism and differences in the toxicity occurrence (Chang, 2006). The depletion of these enzymes will elevate the levels of oxidants in the tissues leading to hepatic injury and other organs damage.

Actually, cisplatin is one of the effective anticancer agents which have been used in the management of many types of solid cancer. Similar to the other antineoplastic agents, cisplatin has severe toxic side effects which prevent its ideal therapeutic effectiveness, especially nephrotoxicity and hepatotoxicity. The nephrotoxicity of cisplatin has been determined as a most common dose limiting factor; but there is no sufficient information about cisplatin-induced hepatotoxicity. Actually, hepatotoxicity can be seen when cisplatin was given in high doses, so cisplatin-induced hepatotoxicity is not considered a dose limiting factor (Zicca et al., 2004). Cisplatin-induced hepatotoxicity is mainly occurred through the oxidative stress mechanism in which the mitochondrion is acting as the first target for cisplatin-induced oxidative stress that are resulting in exhaustion of the mitochondrial protein-SH, reduction in the calcium influx, and a decrement in the

mitochondrial membrane potential (Saad et al., 2004). This mitochondrial dysfunction is considered as a major cause leading to cellular damage.

Tempol is a one of the antioxidant nitrous compounds' group that has been studied significantly in animals with elevated reactive oxygen species (ROS). It is known that tempol shows vasodilatation effects on hypertensive animals (Wilcox & Pearlman, 2008). Tempol is more efficient than other anti-oxidants and even more effective than vitamins. By making comparison between tempol and other nitroxides members, tempol has the same superoxide dimutase (SOD) mimetic efficacy in vitro; but in vivo, tempol is characterized by a highly promotion of scavenging free radicals.

Silymarin is a falvonoid complex extracted from herb milk thistle which is used in the management of liver diseases (Naveau, 2001; laekeman et al., 2003). Its efficacy is relied to its capability of eradicating free radicals and binding with metal ions (Borsari et al., 2001). The frequent indication of silymarin is the liver diseases where it could protect liver cells by inhibition of lipid peroxidation and prevention of glutathione depletion that could result in stabilization of the membrane permeability (Mira et al., 1994; Vanzuela et al., 1989).

The aim of this study is to make comparison between protective effects of tempol and silymarin against the cisplatin-induced hepatotoxicity. The secondary aim of our study is to elucidate the understanding of mechanism of cisplatin induced hepatotoxicity.

4. GENERAL INFORMATION

4.1. Hepatocellular Injury

Hepatocellular injury can be described as a damage of the liver tissues which is related with impaired hepatic function caused by exposure to a drug or another noninfectious agent (Navarro, 2006).

4.1.1. Risk Factors

Generally, history of liver disease does not provoke developing drug induced hepatotoxicity; but some exceptions had been determined in some patients like methotrexate, sodium valproate and aspirin (Brok et al., 2006).

Elder patients tend to be more susceptible to develop drug induced hepatotoxicity due to their high drug usage rate and low metabolism rate. It is more susceptible in patients older than 35 years of age and tends to be more severe in patients older than 60 years.

On the other hand, researchers found the drug induced hepatotoxicity is more likely to occur in female than male especially with nitrofurantoin, isoniazid, flucloxacillin and halothane. Moreover, amoxicillin-clavilanic acid has been reported to cause cholestatic jaundice in males more than in females.

Genetic individual variation can determine drugs metabolism pathway which may facilitate the drug induced hepatotoxicity. There are some thoughts suggesting that genetic predispositions to allergy from some drugs can be considered as co-factor in liver disorders.

Enzyme induction by some drugs like alcohol, rifampicin, paracetamol, halothane, and the combinations of anticonvulsant therapy can potentiate liver toxicity. Actually, this phenomenon can be seen specifically when NSAIDs are used. The risk of hepatotoxicity with NSAIDs is low when used alone; but this risk is highly increased when used with other hepatotoxic agents (Brok et al., 2006).

The co-existence of some circumstances can increase the hepatotoxic activity of some drugs and result in a higher liver damage like in cases of pregnancy, malnutrition, renal disease and diabetes (Brok et al., 2006).

4.1.2. Etiology

According to Gomez-Dominguez (2006), drug induced hepatotoxicity could be an acute attack that could either stay in acute phase or transformed from acute to chronic disease insidiously. The lesions may be described as cytotoxic or cholestatic. The cytotoxic lesion means that hepatic cells damage is occurred; but cholestatic means that there is impairment in the bile flow within capilleries. The mechanisms that lead to hepatic injury can be classified either as:

- Type A: intrinsic hepatotoxicity.
- Type B: idiosyncratic hepatotoxicity.

The intrinsic mechanisms have several properties like the causative drug that has a predictable dose and hepatic injury symptoms can be seen within an incubation period from hours to several weeks. This type of reaction can be reproduced in animals with hepatotoxicant agent like paracetamol, tetracycline, methotrexate and salicylate and others that are shown in the Table 4.1. Actually, toxicity can be avoided by taking the drug within its safe dose without exceeding the toxic dose (Brok et al., 2006).

The idiosyncratic type take place with low incidence rates at a ratio less than 1% in all cases of drug induced hepatic disease. In this type, the injury is less predictable, the severity is not dose dependent, and the incubation period is variable ranging from weeks to months. Due to these characteristics, this pattern of lesion cannot be reproduced easily in animals. However, most of cases are either associated with drug hypersensitivity or metabolism abnormalities. Examples of drugs that can produce this type of hepatic injury are halothane, isoniazid, and chlorpromazine (Gomez-Dominguez et al., 2006).

Table 4.1. Hepatotoxicant Medicines and Their Doses (Martínez et al., 2001)

Drugs	Toxic Dose
Paracetamol	> 10 g as single dose
Methotrexate*	> 15 mg / week > 2 g cumulative dose in 3 years
Tetracycline**	> 2g daily
Vitamin A	Chronic use of 40000 units /day
6-Mercaptopurine	> 2.5 mg/kg
Salicylates	> 2 g / day (chronic use)
Cyclophosphamide	> 400 mg/m2/day
Anabolic steroids	> 1 month (abuse)
Iron	> 1 g (as single dose)
Oral contraceptive***	Duration of the treatment is very important factor

:** The toxicity can be exacerbated with alcohol, diabetes miletus, liver disease; *:** Toxicity exacerbated with pregnancy and renal disease; *****:** The toxicity related mainly to content of estrogen.

4.1.3. Types of Hepatic Injuries:

Hepatic injuries can be divided according to its site, its pathophysiological mechanism, and microscopic pattern as shown below:

Centro-lobular necrosis

The centro-lobular necrosis is usually a dose related, occurring mainly as a secondary reaction to the drugs or as idiosyncratic reactions like in the case of aspiration of halothane. Centro-lobular necrosis can be produced as a result of production of toxic metabolites in addition to the direct effects of drugs. Hepatic injury occurred mainly in the center then diffused to the lobes (Fernandes et al., 1999).

There is two degree of centro-lobular necrosis, the first degree is mild associated mainly with low elevation of liver enzymes and mild degree of liver necrosis and it is described as self-limiting and the second degree tends to be more severe with symptoms of nausea, vomiting, abdominal pain and jaundice with significant elevation of liver enzymes.

This type of liver injuries can be caused mainly by specific types of drugs especially when taken in high dose. As an example, paracetamol can be transformed to a toxic metabolite called N-acetyl-P-benzoquinone imine (NAPQI) which has high affinity to sulfahydryl group.*In vivo*, the sulfahydryl groups in the hepatocytes are regulated by an amino acid called (glutathione), when a high concentration of (NAPQI) is produced in the body; the sulfahydryl group quantity will be insufficient to manage the cytotoxic effect of (NAPQI). At the beginning of the toxicity, the person can reveal mild symptoms like nausea and vomiting with no elevation in the liver enzymes until 40-50 hours later. In this case, N-acetylcystine is administrated for refreshing the hepatocyte's sulfhydryl group storage as soon as possible (Bluckley et al., 1999).

Seatohepatitis

The steatohepatitis is a deferential type of hepatitis which is also called acute steatonecrosis thatis resulting from agglomeration of fatty acids and fats in the hepatocyte.

Steatonecrosis is caused by drugs and/or their metabolites which affect the fatty acids oxidation in the mitochondria.Thereby, the mitochondria will be filled

with fats and will result in hemostasis disruption then cell damage (Lewis J.2000). Many cases of drug induced steatohepatitis represented in the examples below:

- Alcohol is the main cause of steatonecrosis of the liver because alcohol is metabolized to acetyldehyde which exacerbate the fatty acids production, as a consequence, the vessels will be blocked and the vesicles will be damaged resulting in the passing of fats and fatty acids into the blood (Trivedi 2005; Eland 1999). At this case, an inflammatory response will be initiated also and resulting in necrosis of the damaged cells (Bohan et al., 1989).
- This situation can also be seen in patient who is administrated a high dose of tetracycline (1.5 mg/day i.v.). 70-80% of cases have high risk of mortality whereas the survivor will suffer from cirrhosis (Lee 1993).
- Valproic acid also lead to fatty acid accumulation where the cytochrome p450 converts the valproate to delta-4valproic acid which is a very potent inducer of fat accumulation in the hepatic microvascular leading to steatohepatitis (Konig et al., 1999)

Phospholipidosis

The phospholipids accumulation will be occurred in lysosomal bodies of the hepatocytes like in case of amiodarone and its metabolites (desethyl amiodarone). Drug effect on the liver will remain even after discontinuation of the treatment, especially when it is used more than one year. Thus, the patient will suffer from high aminotransferase level, hepatomegaly and even jaundice can be seen (Lullman et al., 1975).

Generalized hepatocellular necrosis

Generalized hepatocellular necrosis is similar to the changes that are seen in the viral hepatitis. The bioactivation process plays a major role in the toxic hepatitis development; but it is not consider the direct cause of it (Beane, 1993). There are many drugs associated with toxic hepatitis bytriggering an immune reaction through binding to a specific cell receptor. The activity of these receptors is related to the genetic polymorphism that can be varied from male to female and from patient to another (Bohan et al., 2002; Liddle et al., 2002; Evans et al., 1999).

Toxic Cirrhosis

This type of cirrhosis can be described as mild. Its development occurred due to the scarring effect of hepatitis. Sometimes toxic cirrhosis is misdiagnosed with viral hepatitis, and the case will be more complicated if the agent or drug is not discontinued. Hepatotoxicity is majorly associated with chemotherapies like methotrexate. The probability of toxic hepatitis can be reduced by the dose intervalincrement (Leonard et al., 1987; Leo et al., 1999).

Cholistatic injury

This type of injury affects the canlicular system which is defined as cholistatic injury. In this type of injury, some drugs may change pericanalicular microfilament network (F-actin) and result in prevention of bile movement within caniculus. The non-removal of the bile from the liver will lead to an intrahepatic accumulation of the toxic bile acids which may cause progressive destruction of liver and results in bile duct syndrome (Cullen, 2005).

Moreover, cholistatic injury can be described either as acute or chronic disorder. Most of patients are seen as cholestasis hepatitis and complain from nausea, malaise, jaundice and prutitis (Dipiro, 2008).

Many causes could lead to choliastic injury like chlorpromazine which is considered the prototype drug which causes cholistatic injury. In addition, vitamin E can cause cholestatic jaundice when given intravenously. Also, total parenteral nutrition (TPN) administration for more than one week can lead to cholestatic injury and to changes in nonspecific enzymes. Patients with low serum albumin are more susceptible than other for this reaction (Lewis 2006; Navarro, 2006).

Mixed hepatocellular and cholistatic injury

In this type, the disease may begin either as hepatocellular or cholestatic and it might spread later. All liver parts are susceptible; but in some cases the disease will affect any part of the liver regardless its location (Dipiro, 2008).

Liver vascular disorders

This type is almost seen in patients who are treated with antineoplastic agents, sex hormones and the pyrrolizidine. It occurs as focal lesion in portal vein, hepatic venules or sinusoids or as central necrosis and then cirrhosis. Herbal tea could cause this type of cirrhosis because they contain comfrey which is a derivative of pyrrolizidine alkaloids (Soe, 1994).

4.1.4. Pathophysiology

Pathophysiology and disease circumstances can be described according to one of these mechanisms that shown in the table below:

Table 4.2. Pathological Mechanisms which is leading to Drug Induced Heapatic Injury (Dipiro, 2008)

Type of Mechanism	*Examples*
Stimulation of Autoimmunity	halothene, sulfamethoxazole
Idiosyncratic reactions	isoniazid, ketoconazole
Disruption of calcium hemostasis and cell membrane injury	lovastatin, venlafaxine
Metabolic activation of the cytochrome p450 enzymes	furosemide, diclofenac
Stimulation of apoptosis	acetaminophen
Mitochondrial injury	aspirin, valproic acid
Liver neoplastic disease	androgens, estrogens

4.1.5. Assessment

The patient history is the most efficient way to evaluate and monitor drug-induced liver disease (Lee W.2003). Patients should fill a questionnaire regarding his drug's use and its concomitant medical history too. Small details should not be ignored like use of cocaine which has a direct effect on liver disease (Van Thiel 1992). Moreover, the toxic effects of street drugs can be exacerbated by concomitant injection or digestion of adulterants because many of them are either toxic or synergies the toxicity of the drug.

On the other hand, non-drug agents are considered as hepatic toxicant and must be determined. For example, Arsenic is on the top list of insecticides that is applied to agriculture; but under low concentration. It is supervised regularly by occupational safety guidelines and health administration protocols that can reduce arsenic toxicity risk; but cannot eliminate it. Actually, Arsenic can cause acute and chronic liver disease if there is a daily exposure to the toxins. This factor can predispose the hepatic reaction when drug is added. Table 4.3 (Dipiro, 2008) below shows some of hepatic toxicant listed in the occupational environment and described as a risk factor for hepatic disorder (Wang 1998).

Table 4.3. Some of Environmental and Occupational Hepatotoxins (Dipiro, 2008).

Hepatotoxin	***Associated Occupations at Risk for Exposure***
Arsenic	Chemical plant, construction workers, farmers
Carbon tetrachloride	Chemical factories technicians, laboratory crews
Copper	Plumbers, painters and artists, copper miners' workers
Dimethylformamide	Chemical factories technicians, laboratory crew
2,4-Dichloro phenoxyacetic acid	Horticulturists
Fluorine	Chemical plant workers, laboratory crews
Toluene	Chemical plant, farmers, laboratory technicians
Trichloroethylene	Printers, dye workers, cleaners, laboratory crews
Vinyl chloride	Plastics factories workers; also seen as a river pollutant

On the other hand, alternative medicines like herbal remedies should also be highlighted in this field due to their important role in the hepatic damage. For example, Chinese remedy *jin bu huan* and also comfrey tea which are famous in market are in reality a common factor that lead to hepatic damage. However, the case of grease wood which is formulated as capsules, the pennyroyal oil, clove oil, and

margosa oil are considered agents causing severe disability or mortality as result from fulminant hepatic failure (Steadman, 1998).

In addition, the nutritional status of a patient could affect significantly in the drug induced hepatic injury aside from the effect of the toxin itself (Jones, 1999). As an example, patients who are malnourished due to diseases or high intake of alcohol will suffer from severe hepatic disease (Seef, 1986). Also, asymptomatic transaminases elevation is almost associated with deficiency of vitamin E, vitamin C, lutein and alpha and beta carotenes. In contrast, when patients have high levels of serum iron, transferrin, and selenium asymptomatic transaminase elevation will be expected (Ruhl, 2003). According to Whitcomb DC (2006), it very important to note that drugs are not the common cause of liver enzymes elevation. For example, a study done in United Kingdom applied on patients admitted to hospitals because of liver enzymes elevation, only 9% of whole cases were resulted from drugs other than alcohol (Dipiro et al., 2008)

Enzymes elevation pattern cannot determine the type of hepatic lesion because the enzymes are not related specifically to the liver (Andriulli, 1998; Paran, 2000). The specificity of any enzyme is related mainly to its distribution extent in the body. For example, alkaline phosphatase can be found in the bile duct epithelium, kidney, intestine and bone. However, 5-Nucleotidase gives more specific results for hepatic diseases than alkaline phosphatase because the body stores this enzyme in the liver. In addition, glutamate dehydrogenase can be used as indicator for centro-lobular necrosis because it is found mainly within centro-lobular mitochondria. Most of hepatic cells have very high concentration of transaminase. Thus, high concentrations of alanine aminotransferase (ALT), aspartate aminotranferase (AST) in serum are indication to their liberation from cells due to severe damage occurred that require weeks to return to its normal level (Paran, 2000).

The serum bilirubin level can be described as sensitive indicator of hepatic damage and used as prognostic evaluation tool. High bilirubin concentrations are related mainly with poor survival. Liver damage will affect to a great extent the secretion of coagulation factors like prothrombin. Thus, poor prognosis can be detected when prothrombin time increased and reach about 40 seconds. Liver transplantation is recommended when encephalopathy or prolonged jaundice are present. Prothrombin time can give an early alert for hepatic disease; but it can

interfere with extrahepatic disease. Thus, the administration of 10 mg vitamin K can recognize between hepatic and extrahepatic disease (Karakoyunlar, 1999).

In fact, liver enzymes and bilirubin measurements give a value of liver condition; but without accuracy about liver function which can be reflected by other clinical tests like serum protein assay (albumin and/or transferin) (Am. J. Cardiol 2001). When hepatic function is defected, serum protein concentration will decreased proportionally and this decrement will be affected by the elimination rate of that protein (Dipiro, 2008).

4.1.6. Management

If the clinical signs and/or laboratory results show hepatic failure, hospital admission is mandatory.

After withdrawal of the causative agent, efforts should be done to remove it from the body. This attempt is beneficial only for acute cases such as in paracetamol, metals, and mushrooms. If patients admitted to the hospital after few hours of ingestion, unabsorbed remedies can be removed by gastric lavage and this method is more preferred than emetics (Brok et al., 2006). The below protocol is considered the corner stone of the liver toxicity management which include several steps to relieve the symptoms and complications. The main therapies include the following:

1. ***Antidotes*:** There are specific antidotes which are indicated for determined toxicants. As an example, acetylcysteine and methionine are used for paracetamol, and desferrioxamine is intended for iron over dose. The desferrioxamine can be given orally directly after oral ingestion and parenterally to chelate the absorbed iron in the blood. The parenteral desferroxamine is indicated when the plasma iron level exceeds 89.5 Mmol/L; but when the plasma level exceeds 62.6 Mmol/l with evidence of presence of free iron, sign and symptoms of acute iron poisoning are present (Brok et al., 2006).

2. ***Corticosteroids:*** Immuno-supression via corticosteroids has been indicated in the management of drug induced hepatic disease to reduce the inflammatory response that may exacerbate live damage; but its usage should not affect

survival of the patient or deteriorate patient's prognosis. In this type, the treatment should be done for a short period to avoid any undesirable effects of steroids (Am. J. Cardiol, 2001).

3. ***Supportive treatment:*** Supportive treatment is a wide term and there are no specific processes for most of patients. Generally, it is suitable for patients with liver failure, with close follow up and correction to fluids and electrolytes levels.

4. ***Pruritis treatment:*** The main differential diagnosis of cholestasis is pruritis which is resulted from accumulation of bile acids in the body tissues. The topical management generally includes avoidance of heavy woolly clothes, skin cooling by calamine or tepid bath, and skin moisturizing with aqueous cream. However, in severe cases systemic treatment is recommended which involves the following:

a. *Anion exchange resins like cholestyramine and colestipol*: These agents act by binding bile acids with resins within gastrointestinal track (GIT) to minimize bile acids reabsorption and enhance their excretion. Resins administration should not be accompanied by other drugs to avoid any pharmacokinetic interaction which may lead to reduce drug absorption. Therefore, any other drug administration must be 1 hour before or after 4-6 hour of resins intake.

b. *Antihistamines:* used to relieve symptomatic itching; but the sedative antihistamine use is contraindicated in liver disease because it may mask the symptoms of hepatic encephalopathy.

c. *Opioid antagonist*: It is used to reduce the feeling of the discomfort due to itching sensation.

5. ***Rifampicin and phenobarbital:*** They can induce cyto P450 enzymes and increase bile flow. Rifampicin has a potential hepatotoxicity activity so it must be used with caution to avoid any deterioration of liver failure.

6. ***Coagulation disorders correction:*** It occurred mainly due to vitamin K deficiency and it is can be elevated through phytomenadione i.v.

administration. Within 3-5 days the prothrobin time will be corrected. Oral phytomenadione (lipid soluble form) intake is not effective because it will not be absorbed from GIT. On the other hand, menadione (water soluble form) tends to be more absorbable. If patient admitted to emergency ward with bleeding, fresh frozen plasma and/or clotting factors should be applied immediately.

7. ***Long term treatment:*** When drug induced hepatic disease diagnosed and its management is started, the treatment of the main disease for which the causative agent is prescribed should be considered. In many cases drug treatment is continued with alternative agents; but more caution and follow up are recommended, especially when the alternative agent has the same chemical properties or same side effects profile.

4.1.7. Role of Pharmacist

In fact, pharmacist should inform the patient about the risk of over dose of drug and should make awareness about formulations containing the same causative agent. For example, paracetamol can be an ingredient within more than one formula. Almost all patients should be educated about the possible side effects and this information must be confirmed by medication information leaflet. Patients and their caring crew must be able to recognize the symptoms that are related with liver disease and reported them directly like malaise, nausea, fever and abdominal pain. Although these are non-specific symptoms; but they must be taken into consideration and liver function tests must be tested, when these symptoms accompanied with high liver values, the drug must be stopped immediately (Brok et al., 2006).

After recovery, patient should be aware about the causative agent of the side effects in order to avoid future prescription containing past medical history taken. The big challenge may encounter the pharmacist when informing the patient the potential side effects without creating non-compliance to the vital medications. In this case, periodical liver function tests surveillance is recommended for patients who are more susceptible to liver disease like those with past history, or taking more than one liver toxicant medications, or are over 40 and heavily alcoholic patients. Thus, in those patients, the follow up should be done every 2 months. Whereas, in case of low risk patients, the tests are done when signs or symptoms are presents like

(fever, malaise, vomiting, jaundice or unexpected flare during treatment) (Cardiol, 2001).

Finally, liver function tests monitoring is not the key way to prevent liver injury because many cases may develop within very short duration and the elevation of liver enzymes is not always an indicator for probable fibrosis (Martínez et al., 2001).

4.2. Tempol

The chemical name of Tempol is (4- hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl). It is one of nitric oxide that had been studied extensively. Tempol improves the hydrogen peroxides metabolism by action that looks like catalase action, and reduces the production of toxic hydroxyl radicals (Christopher, 2008).

4.2.1. Interaction of Tempol with Reactive Oxygen

Nitroxides are termed ”Super Oxide Dimutase (SOD) mimetics” because they catalyze conversion of $O_2^{\bullet-}$ into H_2O_2which is easily metabolized by the liver. Actually, tempol catalyze metabolism of $O_2^{\bullet-}$ which is generated by xanthine and xanthine oxidase or by angiotensin II metabolism (Chateauneuf et al., 1988; Luo et al., 2007). The transformation of nitroxides to hydroxylamine occurs mainly in the cells reversibly. This reaction is accelerated by presence of ascorbate in erythrocytes and liver. The dehydroascorbate is resulting from ascorbate oxidation by the action of tempol. Furthermore, ascorbate is a best reducing agent in the erythrocyte (Bobko et al., 2007).

Nitroxides such as tempol can detoxify, metabolize, minimize or stop formation of wide range of other ROS like H_2O_2. Nitroxides can decompose or prevent formationof OH•, singlet oxygen, peroxyl radicals, nitroxyl anion, peroxynitrite, and nitrogen dioxide which are generated by myeloperoxidase radicals, and peroxidation of lipid products or phospholipid. Nitroxides could prevent any damage of tissues resulted from oxidizing of the reduced transition metals forms like ferrous and cuprous ions or cadmium or chromium (Samuni et al., 1991; Borisenko et al., 2004).

In fact, the role of tempol is protecting lipid, DNA and proteins from oxidative damage, and can be interacted with other antioxidants to promote their action in the reduction of oxidized lipids. Furthermore, nitroxides can prevent the damage that may result from the oxidation in cells or organs like in the skin when exposed to UV radiation. Moreover, tempol has protective activity to cells targeted by X radiation (Damiani et al., 2006; Shen et al., 2006).

There are several standards to qualify the activity of antioxidants like IC50 values of nitroxides in preventing the lipid's peroxidation by •OH (the value can be assessed by MDA production in the tissue stimulated by Fe^{2+} with ascorbic acid), preventing damage of the cells by H_2O_2 (evaluated by red blood cell hemolysis by H_2O_2), and facilitating O• metabolism (evaluated by formazan generation by adding nitroblue tetrazolium to zymosan A-stimulated leukocytes). As a result of comparison among 8 derivatives of nitroxides, Tempol had less IC50 activity and high activity to prevent the formation of •OH from H_2O_2 (which is done by hemolysis) or high metabolic activity on $O^{\bullet 2-}$ (which is considered SOD assay) (Takeshita et al., 2002).

4.2.2. Pro-Oxidant Actions

Tempol administration in high concentration can lead to pro-oxidation effects especially on the smooth muscles and endothelial cells. This paradoxical activity occurred mainly due to its dual ability in $O^{\bullet 2-}$ initiation and finishing the lipid peroxidation (Offer et al., 2000). This finding may explain the ability of tempol to protect the cells in low concentration and enhancing the cell toxicity in high concentration. Generally, the pro-oxidant activity can be prevented by addition of other antioxidants like ascorbate (May et al., 2005).

4.2.3. Antihypertensive Activity

Tempol had been reported as antihypertensive agent, its activity can be described as acute and prolonged active agent. Tempol antihypertensive action is contributed to its ability to reduce heart rate (Patel et al., 2006) and its action on renal-sympathetic nerve activity (Xu et al., 2004). When tempol given intravenously rapid fall of blood pressure can be recognized; but this action be reserved by the

body rapidly, tempol when given oral its action can seen after several days and its duration may continue over longer time (Christopher, 2008).

4.2.4. Metabolism and Pharmacokinetics

Nitroxides are considered stable compounds with low plasma protein binding (Okajo et al., 2006). Nitroxides reveal characteristic properties in their pharmacokinetics due to the existence of a single unpaired electron. Therefore, nitroxides can be detected by magnetic resonance imaging (MRI) (Hyodo et al., 2006; swartz et al., 2007). The main pathway of tempol metabolism is conversion to hydroxylamine by the liver, and this pathway is done by NADPH and cytochrome C (Lanne at al., 1989).

After ingestion, tempol can be distributed all over the body and finally reaches into the cells. Within the cell, tempol reacts with the $O^{\bullet 2-}$ in the cytoplasm and mitochondria (Paul et al., 2006). Furthermore, tempol could pass the blood brain barrier (Behringer et al., 2002) and penetrate the intact skin and be accumulated within the lipid compartment of the stratum corneum (Lie et al., 1999). Tempol could reach the underlying bone via cartilage (Fischer et al., 1995). When Tempol is administrated intravenously, it can reach the bile system directly and may increase the hepatic uptake of bile acids and also increase biliary excretion (Hahn et al., 1998).

4.2.5. Tempol Toxicity

The toxicity of tempol have been studied and documented, these studies that concerned with this subject showed the LD50 intraperitoneal dose of tempol is 1.6gm/kg i.e the LD50 dose is 30 fold more than minimum effective dose (Hahn et al., 1992).

The toxicity symptoms of tempol can be seen after 20 minutes from intraperitoneal injection and involve mainly restlessness and seizure. It is reported also that tempol can reduce the osmotic fragility of red blood cells and lead to its destruction which may result in hemolysis (Bieri, 1974).

4.3. Silymarin

Silymarin is an antioxidant described as flavonoid complex and extracted from *Silybum marianum (L.)* and has been currently used in the treatment of liver disorders (Naveau, 2001; Laekman et al., 2003). This ability as known comes from its capability of scavenging free radicals and chelating metal ions (Borasari et al., 2001). Silymarin is able to protect liver tissues mostly through stabilizing the liver cells' permeability by reduction of lipid peroxidation (Mira et al., 1989) and by compensating liver glutathione depletion (Valenzuela et al., 1989).

4.3.1. Herbal Use

As commonly known, milk thistle which is called (silymarin) is used for disease of liver, gall bladder and spleen. It is also can be used as milk production stimulator in lactating mothers, for hemorrhoids and, for dyspepsia. It is also used as demulcent in pleurisy and catarrh (Morazzoni, 1995; Awang, 1993).

Most of the medical interests are focusing on the protective and curative action of milk thistle toward liver damage.

4.3.2. Pharmacological Actions

The documented pharmacological activities for thistle fruit include antioxidation, hepatoprotection, antifibrosis, anti-inflammatory, anti-tumor, anti-lipid peroxidation. It is also described as stimulant of protein synthesis and enhancement of liver regeneration. Silymarin is normally considered as mixture of isomers of silibin, silichristin and silidianin, and silymarin play the most active pharmacological role in the thistle fruit (Morazzoni, 1995). Actually, silibin is the main component of silymarn. The well-known effects of silymarin have always related to the hepatoprotection action; but also there are several effects that are described as below:

Antioxidant:

Silymarin is an antioxidant reacting with free radicals and making them more stable and less reactive molecules (Morazzoni, 1995; Fitoterapia, 1995). Silymarin reduces the lipid peroxidation which is accelerated by iron linked system in rat liver

microsomes. Moreover, Silymarin protects erythrocytes by inhibition of phenylharazine induced lipid peroxidation (Morazzoni et al., 1995). However, when silyamrin is given to rats intraperitoneally can cause elevation of glutathione in liver, intestine and stomach and results in more production of sulfhydryl group (Valenzuela et al., 1989). Also, it has been reported that silymarin could protect blood vessels through prevention of copper-induced oxidation of human low density lipoprotein (LDL) (Skottova et al., 1999).

Hepatoprotective activity:

In vitro studies show that isolatedhepatocytesand some of its components can be protected by using silymarin against damage caused by various cytotoxic factors (Morazzoni and Bombardelli, 1995).

A lot of *in vivo* studies had been done demonstrating the hepatoprotection action of silymarin in acute and chronic liver disease. The acute cases of liver disease majorly induced by several toxic agent like carbon tetrachloride, thioacetamide, paracetamol, ethanol, thallium, alpha - amantin and phalloidin, ; but most of the chronic cases aredue to chronic exposure of liver toxicant agents which include carbon tetrachloride, heavy metal, thioacetamide or several drugs like indomethacin and azathioprine. Other studies show also that silymarin has potency against liver damage caused by gamma radiation and ischemia (Kropacova et al., 1998). Also it had been reported that silymarin has a hypocholesterolaemic and ability to increase bile acids secretions from liver (Krecman, 1998; Crocenzi, 2000).

Anticancer activity:

Silymarin have shown anticancer activity when given topically to mice exposed to tumor provokers' 12-O-tetradecanoylphorbol (TPA) and okaidic acid (OA) (Zi X et al., 1997). In this manner, Silymarin have ability of inhibitionfor tumor necrosis factor alpha (TNF-α) induction when given prior to tumor inducers (Lahiri-Chatterjee M et al., 1999).

Silymarin exerts a potent anticarcinogenic activity against prostate cancer and reduces prostate-specific antigen (PSA) in hormone-refractory human prostate cancer and prohibits cell growth (Zi X et al., 1999).

In low doses of silymarin exerts a significant action as anticarcinogenic against human breast carcinoma cells (Zi X et al., 1998).

Anti-inflammatory activity:

When silymarin is given intraperitoneally to mice, it will lead to inhibition of leukocytes aggregation in the inflammatory exudates and reduce neutrophil cells count. Also, silymarin could inhibit mitogen-activated protein kinase and c-Jun N-terminal kinase and suppress COX-2 and IL-1.In addition, while making comparison between indomethacin and silymarin. Silymarin has greater potency toward in xylene induced inflammation (Zhao et al., 1999).

Gastric ulcer protective effects:

Silymarin could prevent stress-induced ulcer through reduction of histamine secretion and inhibition of the enzymatic peroxidation by lipoxygenase pathway (De La et al., 1996).

4.3.3. Clinical Studies

Clinical trials with milk thistle preparations have focused on their activity against alcoholic liver disease, cirrhosis, acute and chronic viral hepatitis. There are trials that have included patients suffering from liver disease caused by different etiology like alcoholic and non-alcoholic cirrhosis (Joanne, 2002).

4.3.4. Pharmacokinetics

Studies had been done to investigate the pharmacokinetics properties of silymarin and its components in healthy persons and patients suffering from cirrhosis and those who have cholecystectomy, the results show that around 20-50% of silymarin is absorbed by GIT. In addition, when silymarin administrated orally or intravenously, 80% of the dose will be excreted through bile duct (Mennicke, 1975).

4.3.5. Side Effects and Toxicity

In pharmacokinetics studies, healthy volunteers were exposed to a single dose of 254 mg silymarin and the researchers did not note any adverse effects following this dose (Lorenz et al., 1984). Actually, a case is reported in Australia where the patient was suffering from severe sweating, abdominal cramping, nausea, vomiting, diarrhea and weakness as a result of silymarin reaction (Anon, 1999). Moreover, in another case, anaphylactic shock was reported in a 54 years old man suffering from kiwi hypersensitivity who experienced facial edema, bronchospasm, oral mucosa swelling, respiratory distress, and decreased blood pressure after taking preparation of silymarin (Geier et al., 1990).

4.4. Cisplatin

Cisplatin is known as one of the most effective anti-neoplastic agents used in the treatment of cancer. Actually, cisplatin administration was accompanied by many side effects like nephrotoxicity, ototoxicity, neurotoxicity, myelosuppression and severe nausea and vomiting. Most of these side effects are considered clinically significant especially nephrotoxicity. However, hepatotoxicity is less significant than the above events and not considered as dose limiting factor; but it can be more serious when cisplatin is combined with other hepatotoxicant agents or administrated in circumstances that can increase its toxicant activity (Barabas et al., 2008).

4.4.1. Pharmacokinetics

Plasma cisplatin has been shown to be protein bound. In contrast, intracellular cisplatin appear to be protein free and can be distributed all over the body tissues (Jacobs et al., 1980; Choie et al., 1980). After one hour from its intravenous injection, cisplatin will be accumulated in liver, kidney, skin, and muscles. Cisplatin accumulation can be delayed in liver and kidney when given in high doses and it can

be present for more than one week in these organs. Cisplatin concentration tends to be higher in tissues where it can exert its antineoplastic activity such as uterus and ovaries (Litterst et al., 1976).

Some studies showed that pharmacokinetics properties of cisplatin can be changed in cancerous tissues. The tumor can change the distribution time of cisplatin; but has not effect on its elimination from body (Litterst, 1988).

4.4.2. Mechanism of Action

The primary action by which cisplatin exerts its effect is DNA synthesis inhibition. This inhibition can be occurred in low doses in comparison with doses which are required to inhibit RNA synthesis (Harder 1970; Howle 1970).

This mechanism of action depends mainly on aquation of the DNA strands through which two chloride sites will be replaced by two molecules of water. Thus, this effect will prevent DNA strands cross linkage and cell mutliplication will be stopped (Weiss and Poster 1982).

4.4.3. Indications

There are many indications of cisplatin administraton in the treatment of cancer; but the main uses of cisplatin are followed as:

Bladder cancer: Cisplatin in these cases are used as single agent especially indicated for advanced bladder cancer in patients who previously submitted to surgery and local therapy.

Ovarian cancer (metastatic): Cispaltin used in combination with other agents in in patients who no longer get benefits from surgery or local therapy.

Testicular cancer (metastatic): In the treatment of metastatic testicular cancer cisplatin is also used combined with other chemotherapeutic agents after exlusion surgery and other local therapy (www.online.lexi.com updated 16/12/2015).

4.4.4. Mechanism of Resistance

There are four pathways where cancerous tissues can resist cisplatin activity (Reed, 2006). These pathways include:

1. Reduction of cellular accumulation
2. Cytosolic deactivation of cisplatin
3. Increment of DNA repairing mechanism
4. Apoptosis

Cisplatin resistance may be initiated also from covalent binding of sulfahydryl group and proteins with cisplatin. Glutathione (GSH) and metallothionine (MRP2) could have an important role in cisplatin resistance (Borst P 2000).

4.4.5. Toxicity

Cisplatin is associated with many side effects that are limiting its use or lowering its optimum therapeutic activity. These side effects will be more severe and significant when cisplatin is used within combination of other antineoplastic agents to enhance the eradication of cancerous cells (Barabas et al., 2008). The most important side effects of cisplatin include the followings:

Gastro intestinal tract toxicity: This toxicity is occurred mainly due to death of the lining cells of GIT. The activation of chemo-trigger zone in the brain will be revealed as severe nausea and vomiting.

Myelosuppression: It is occurred mainly as a result of bone marrow cells death. The hematologic change is an indicator about its occurrence.

Ototoxicity**:** It is highly common seen in patients who are recieved high doses of cisplatin. Ototoxicity had been seen in 7-90% of patients recieved 120mg/m^2 (Ellerby, 1974; Green, 1972).

Neurotoxicity: Mainly occurred in the peripheral nerves, its symptoms appear as parastesia and severe neurolgia (Ozols and Young, 1985).

Syndrome of inappropriate secretion of antidiuretic hormone (SIADH): This side effect has been reported in the patients with the administration of cisplatin and other cytotoxic drugs (Andersen and Hansen 1995).

5. MATERIAL AND METHODS

All animals are supplied from GATA military hospital in Istanbul province. The experiments are approvedby Marmara University Animal Experiments Ethical Committee referring to the protocol number 56.2014.mar (Attachment 1).

Animals: Adult female Wibstar rats, weighing 250-300 g were obtained from Experimental house of Istanbul military hospital (Gulhane Askarlik Tip Akademisi). They are kept under standard condition with normal diet and drinking water in Marmara University Experimental Animals Laboratory (DEHAMER) within specific cages, each cage contains 3 animals.

Material: Cispaltin, tempol, and silymarin were purchased from Sigma-Aldrich Chemical Co. Silymarin was suspended in distilled water and administrated by oral gavage. Cisplatin and tempol were dissolved in saline and were administrated intraperitoneally.

Experimental design: Animals were divided into 6 groups each of 6 animals; three groups were described as control, one as toxicity and two as treatment groups. The first group of rats were administrated saline orally and intraperitneally as control group. The second group of rats was given cisplatin 10 mg/kg intraperitoneally as a single dose on the seventh day (Palipoch et al., 2013). The third group of animals received regularly along seven days 15 mg/kg tempol intraperitoneally (Babilonia et al., 2005). The fourth group of rats was given 100 mg/kg silymarin orally for seven days (Rajeswara Rao and Viswanath 2007). The fifth group was given tempol 15mg/kg by intraperitoneally injection and cisplatin 10mg/kg as single intraperitoneal dose on the seventh day. The sixth group was given silymarin 100mg/kg by oral cavage for seven days and cisplatin 10 mg/kg by single intraperitoneal injection on the seventh day of experiment.

After 24 hours of cisplatin injection, the experimented animals were anesthetized with ether. Then, the animals had been sacrificed and their livers were removed and washed by ice saline, canned with a piece of filter paper and

homogenization was carried out according to Branson sonifier (250, VWR Scientific) for further investigation

Table 5.1. Experimental Protocol

Experimental Design		
Groups	***Compounds***	***Dosage and route of adiminstration***
Control	Saline	1mL orally + 0.1 mL saline intraperitoneally for 7 days
Control	Tempol	15mg/kg tempol intraperitoneally for 7 days
Control	Silymarin	100mg/kg silymarin for seven days orally
Toxicity	Cisplatin	10mg/kg cisplatin intraperitoneally as a single dose on the 7^{th} day
Treatment	Tempol + Cisplatin	1mg/kg tempol for 7 days intraperitoneally + 10mg/kg cisplatin single dose on 7^{th} day intraperitoneally
Treatment	Silymarin + Cisplatin	100mg/kg silymarin orally for 7 days+ 10mg/kg cisplatin single dose on 7^{th} day intraperitoneally

5.2. Evaluation Methods

5.2.1. Biochemical Parameters

Malondialdehyde (MDA) Analysis

Malondialdehyde (MDA) levels in liver tissue homogenates were determined to detect lipid peroxidation level in the hepatic tissues. This parameter was analyzed by thyobarbituric acid- reactive substance (Ohkawa et al., 1979). At first, in order to get the supernatant, potassium chloride KCl was added to the homogenates and mixed by homogenizer (Janke& Kunkel, IKA - WERK) to get 10% tissue homogenate. The reactive solution was prepared from 0.2 mL of 8.1% of sodium dodecyl sulphate, 1.5 mL aqueous solution of thyobarbituric acid (pH=3.5), and 1.5 mL 20% acetic acid (pH 3.5). Tissue supernatant (0.1 ml) was added to reactive solution then distilled water was added up to 4 mL and heated to 95^0 C for 30 minutes.Then, this mixture was cooled and mixed again with 1.0 mL distilled water and 5 mL of n-butanol-pyridine (15:1) mixture. The obtained mixture was mixed again by vortex to ensure a proper mixing for the constituents and centrifuged under 4000 rpm for 10 minutes. After that, the resultant upper organic layer was taken and analyzed by spectrophotometer at 532 nm. The values obtained were multiplied by 211.5 resulting in concentration of MDA as nmol/g of tissue

Total Antioxidant Status (TAS)

TAS was measured in supernatant of rats liver tissues by using fully automated 3^{rd} generation TAS Assay Kit (Rel Assay Diagnostics®). The dark blue-green colored radical in the kit was reduced by the antioxidants that werepresented in the samples to change its color from dark blue-green to colorless form. Absorbance was measured with spectrophotometer at 660 nm referring to the total antioxidant level represented in the sample. In order for this assay to be done, it should be calibrated with stable antioxidant solution.This standard solution is called Trolox which is a compound equivalent to vitamin E.

Myeloperoxidase (MPO)

MPO activity measurement in liver tissues was done with rat myeloperoxidase (MPO) ELISA kit (Bioassay Technology Laboratory) which is dependent on the biotin double antibody sandwich technology to assess (MPO) levels, homogenates were added to the wells that are coated with (MPO) monoclonal antibodies after that were incubated. Later, we added anti MPO antibodies to streptavidine-HRP to form immune complexes. After incubation, we removed unbound enzymes and washed them, and then we added substrate A and B. After that, the solution turned to blue then ınto yellow by the effect of acidity. The resultant solutions were entered to the ELISA to read them.

5.2.2. Histopathological Evaluation

The hepatic samples were evaluated by light microscope fixed in 10% buffered formalin for 2 days and embedded in paraffin. After that, sections were prepared for morphological examinations by sectioning approximately 4 micrometer thickness and stained by hemathoxylin and eosin (H&E). Sections were observed with photomicroscope (Olympus BX51, Tokyo, Japan) and photos were taken with digital camera (Olympus C-5060, Tokyo, Japan). Histopathological criteria are: a) damaged vacuolated and pyknotic hepatocytes; b) vascular congestion and sinusoidal dilation; c) number elevation of active death kupffer cells. Necrosis score from 0 to 9 was used for histology evaluation (Sener et al. 2005).

5.3. Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 5.0 (GraphPadSoftware, Inc. La Jolla, CA, USA). All data are expressed as means ± SEM. Relationship within and between groups is measured with Mann Whitney U and ANOVA tests by using Tukey test as a post-hoc test. Group differences of $p<0.05$ were considered statistically significant.

6. RESULTS

6.1. Malondialdehyde (MDA) Levels

The measured MDA levels of the hepatic tissues were shown in the Figure 6.1. In the groups of control (saline, tempol and silymarin) values of MDA level showed as following 60.98±8.02 mg/g tissue; 74.66±9.45 mg/g tissue; 93.41±6.2 mg/g tissue respectively. No significant differences have demonstrated among them ($p>0.05$).

In comparison between control saline and cisplatin toxicity group (176.3± 21.55 mg/g tissue), the cisplatin toxicity group shows significant elevation in MDA level, reflecting the oxidative stress of cisplatin on rats' hepatocytes.

However, MDA level value of cisplatin + tempol group was (58.46±5.34 mg/g tissue) and MDA value of cisplatin + silymarin group was (80,65±6.95 mg/g tissue), both of them have exhibited significant decrease of MDA levels in liver tissues due to the antioxidants effects of the used agents (tempol and silymarin) ($p<0.01$).

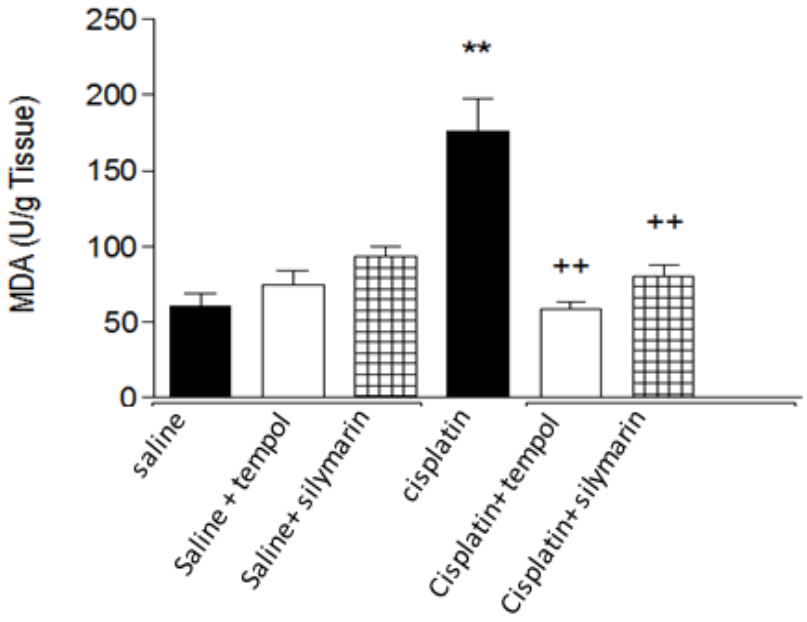

Figure 6.1. Malondialdehyde (MDA) levels in rat liver tissues.

*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, in comparison with saline control group

+: $p<0.05$; ++: $p<0.01$; +++: $p<0.001$, in comparison with cisplatin toxicity group

6.2. Myeloperoxidase (MPO) Levels

The MPO levels have been assessed in rat liver tissues shown in the Figure 6.2. The control groups of saline, tempol and silymarin had MPO values as following: 22.03±0.45 U/g tissue, 23.06±0.83 U/g tissue, 18.27±2.46 U/g tissue, respectively, with no significant differences among them ($p>0.05$).

However, the comparison between cisplatin toxicity group which had a value (31.81±1.67 U/g tissue) and control indicates a significant difference ($p<0.001$).

Tempol and cisplatin administrated group had MPO value (23.19±0.7 U/g tissue), MPO level is significantly low when compared with cisplatin toxicity group. This difference explains the activity of tempol in reducing MPO level ($p<0.01$).

In addition, the effect of silymarin is obvious in reducing MPO level in hepatic tissues. This effect is revealed when comparison is done between cisplatin administrated group and silymarin + cisplatin group (23.37±1.2 U/g tissue), the significant difference reflecting the activity of silymarin ($p<0.01$).

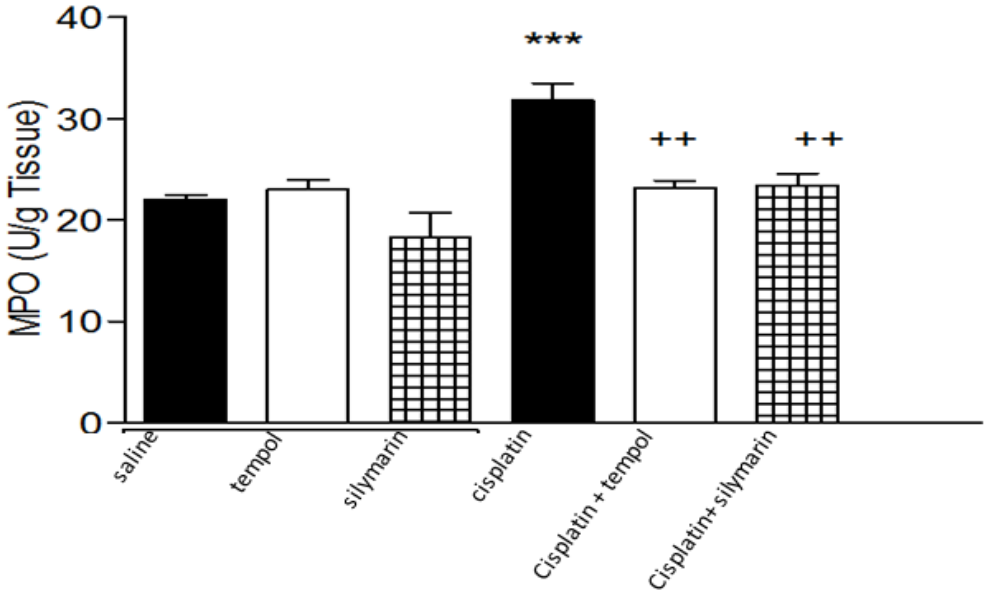

Figure 6.2. Myeloperoxidase (MPO) levels in rat liver tissues.

*****: $p<0.05$; ******: $p<0.01$; *******: $p<0.001$, in comparison with saline control group

+: $p<0.05$; ++: $p<0.01$; +++: $p<0.001$, in comparison with cisplatin toxicity group

6.3. Total Antioxidant Status (TAS) Levels

The measured TAS levels of rats' hepatic tissues are shown in the Figure 6.3. TAS levels of control, tempol control, silymarin control groups (1.658±0.027 U/g tissue, 1.818±0.18 U/g tissue, 1.75±0.15 U/g tissue) appear to be high with no significant differences are detected between them ($p>0.05$).

On the other side, cisplatin toxicity group TAS level (0.4427±0.13 U/g tissue) has appeared with significant low level in relation with control group, this difference explains the lack of antioxidative proteins as a result of cisplatin administration ($p<0.05$).

TAS levels In tempol treatment group (1.652±0.14 U/g tissue) and silymarin treatment group (1.672±0.19 U/g tissue) have expressed significant high levels in comparison with cisplatin toxicity group indicating the capability of these antioxidants in preventing the oxidation process which may occur as a result of cisplatin administration ($p<0.01$).

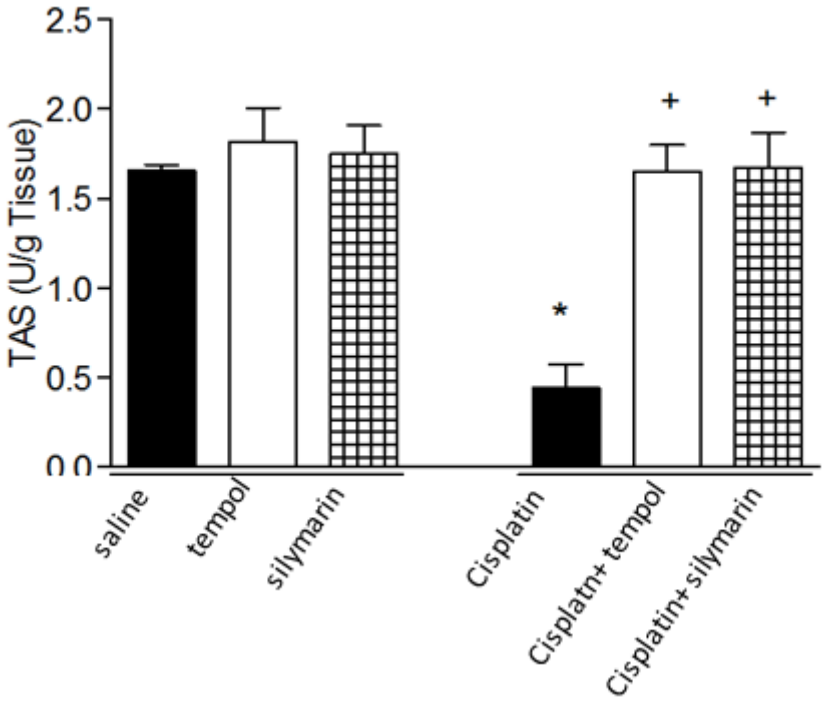

Figure 6.3. Total Antioxidant Status (TAS) in rat liver tissues.

*****: $p<0.05$; ******: $p<0.01$; *******: $p<0.001$, in comparison with saline control group

+: $p<0.05$; ++: $p<0.01$; +++: $p<0.001$, in comparison with cisplatin toxicity group

6.4. Histopathological Evaluations

The histopathological evaluations are shown Photo 6.1. and lesion score values shown in Table 6.1.

Firstly, the parenchyma of control group liver tissues appears to be normal with no abnormal morphological demonstrations.

However, the parenchyma of cisplatin toxicity group shows sinusoidal dilation and congestion, vacuole formation increment with high number of damaged cells and active dead kupffer cells.

Moreover, silymarin and tempol group hepatic parenchyma exhibits significant low level of sinusoidal dilation and active dead kupffer cells in comparison with cisplatin toxicity group tissues.

Table 6.1. Lesion Score Values of The Hepatic Tissues

group	Lesion Degree
Control saline	0±0
Tempol	0.8333±0.54
Silymarin	0±0
Cisplatin	6.667±0.33
Tempol + cisplartin	1.167±0.16
Silymarin + cisplatin	3±0.31

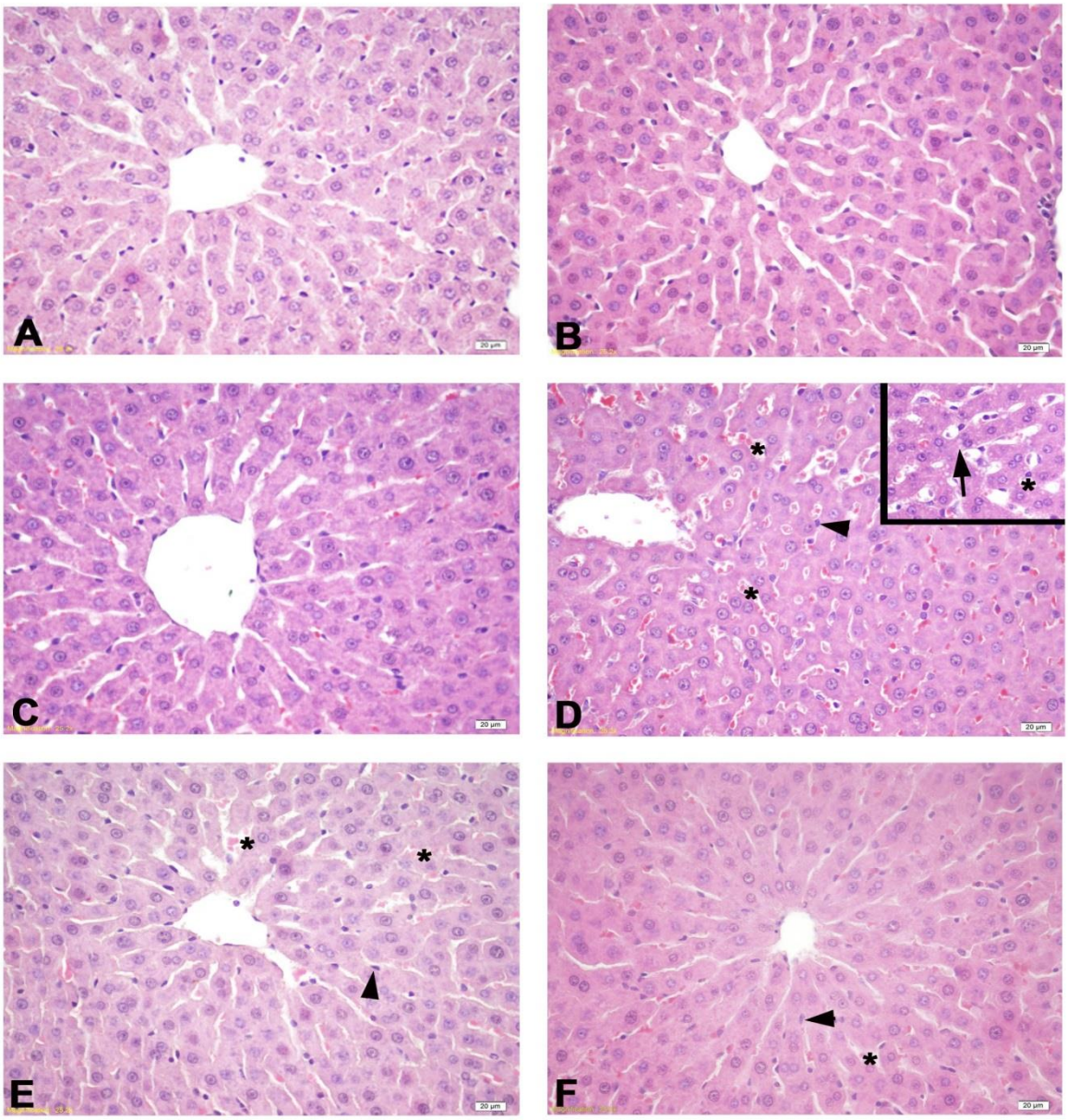

Photo 6.1. Histopathological Examinations Results

A: Control; B: Control Silymarin; C: Control Tempol, in these groups liver tissues seem normal.; D: Toxicity, in this group serious sinosidal dilatataion and congestion can be seen as (*) also vecules are formed with enumerous dead hepatic cells are seen; E: Cisplatin and silymarin; F: Cisplatin and tempol, in these groups, liver tissues appear to be normal morphological critirea with low numbers of dead cells and congestion.

7. DISCUSSION

Many studies had been conducted toward demonstration of cispaltin's effects on kidney (Kersten et al., 1998; Husain et al, 1998; Okuyan 2007); in contrast, few studies evaluated cisplatin effects on liver. Thus, this study is advocated to examine cisplatin destructive impact on liver and effect of administration of antioxidants, tempol or silymarin, as a protective tool of cisplatin induced hepatotoxicity in rats' model. Cisplatin is considered as one of the effective anticancer drugs. Actually, severe toxic side effects like hepatotoxicity are noticed in high doses administration (Zicca et al., 2004; Kim et al., 2004; Koc et al., 2005; Pratibha et al., 2006). To our knowledge, the current study is the first study concerned with the effect of tempol against cisplatin induced hepatotoxicity.

In fact, oxidative stress is a common cause in the initiation and progression of hepatic damage in many types of liver disease. The damage is occurred when there is abundant reactive oxygen and nitrogen species, or corruption in the anti-oxidation mechanism (Medina and Moreno-Otero, 2005).

Our study show an elevation in MDA levels of toxicity group (176.3± 21.55 mg/g tissue) which explains the accelerated peroxidation level in the liver that is in contrast with the treatment groups where MDA was near to normal level due to the effects of tempol and silymarin Figure 6.1. Our study results are in coordinated with other studies (Palipoch et al., 2013; Yongke Lu and Arthur I. Cederbaum 2005). MDA level was significantly decreased in cisplatin with tempol group (58.46±5.34 mg/g tissue) and cisplatin with silymarin treatment group (80,65±6.9 mg/g tissue) due to the oxidation inhibitory mechanism of tempol and silymarin. In other words, tempol and silymarin guard cells against free radicals that are generated by lipid peroxidation through scavenging property.

Referring to Palipoch (2013), TAS levels tend to be low when intrinsic antioxidants proteins are depleted. According to our study, the TAS level appears to be high in control groups (saline, tempol and silymarin) which shown as following: 1.65±0.02 U/g tissue, 1.81±0.18 U/g tissue, and 1.75±0.15 U/g tissue respectively. On the other hand, TAS level in cisplatin group was significantly low. In addition, while combination of cisplatin + tempol and cisplatin + silymarin in treatment groups, TAS levels were significantly higher Figure 6.3. Thus, it is concluded that

the low level of TAS in toxicity group is resulted from cisplatin administration; but the improvement in the treatment groups was obtained due to tempol and silymarin administration.

Studies indicating free radicals are responsible of the formation of activated neutrophils. Actually, in addition to free radicals direct destructive activity on the hepatic tissues, they also can increase the ability of neutrophil to be accumulated in the tissue. After accumulation, it provokes tissue damage through neutrophils activation, and then activated neutrophils will secrete enzymes (eg. Meyloperoxidase, protease) which increase the activity of the oxygen free radical (Omar et al., 1989).

According to our result, MPO values in the hepatic tissues of the rats appear to be higher in the toxicity groups (31.06±1.671 U/g tissue) in comparison with MPO measurement of control groups Figure 6.2. In concordance with studies (Palipoch et al., 2013 ; Kim et al., 2004), our toxicity group had the highest value of MPO as a result of cisplatin effect. However, saline (22.03±0.45 U/g tissue), silymarin (18.27±2.46 U/tissue) and tempol (23.06±0.83U/g tissue) control groups showed no significant differences among each other.

Actually, tempol and silymarin effects can be seen when comprasion is done among MPO levels of cisplatin toxicity group with tempol treatment group and silymarin treatment group, MPO levels in the treatment groups were significanly low Figure 6.2. In other words, tempol and silymarin administration can cause a significant reduction of MPO when cisplatin induce toxicity.

Histopathological scoring is a method which indicate the damage that resulted from toxicant agent (Sener et al. 2005). Our study is in agreement with other studies which indicated cisplatin ability to cause a necrosis and damage in the parenchyma of the liver (Yongke Lu and Arthur I. Cederbaum 2005).

Inflammatory reaction and oxidation which are considered the main cause of tissue damage play an important role in the congestion and vacuole formation in the hepatic tissues. However, our study revealed that cisplatin toxicity group showed a highest score of tissue damage (6.667±0.33) and most numerous dead hepatic cells. On the other hand the score of saline (0.0±0.0), tempol (8.33±0.54), and silymarin (0.0±0.0) control groups with no necrotic cells or tissue damage. Moreover, tempol and silymarin treatment groups revealed very few or no necrotic cells, these

demonstrations indicates the protective effects of tempol and silymarin against cisplatin induced hepatic injury Table 6.1 and Photo 6.1.

Histologically, in the only cisplatin administrated group kupffer cells can be seen in higher density than other groups as result of injury, inflammatory response, and oxidative stress. As it is already known, kupffer cells are the most abundant resident macrophages in tissues which play a specific role in immunity regulation, phagocytosis, and biochemical defense (Decker, 1990). Indeed, Kupffer cells' activation is occured through membrane receptors that mediated release of enzymes, proteins, active lipid, and oxidative stress (Decker, 1990, Wang et al., 1993). Our study is concordant with studies (Palipoch et al., 2013; Yongke Lu and Arthur I. Cederbaum 2005), in which the control groups do not contain kupffer cells; In contrast, in the toxicity group tissues, kupffer cells seems to be more abundant as a result of cisplatin induced toxicity which is due to inflammatory response produced from the oxidation action of cisplatin. Finally, the Kupffer cells population density in treatment groups are significantly lower than cisplatin group and are similar to their level in the control groups. These phenomena indicate the action of tempol and silymarin in reducing the factors that induce kupffer cells appearance.

Referring to Soto (2003), the hepatotoxicity is not caused all times by free radicals only; but it is formed also due to exhaustion of SOD, CAT, and GSH their depletion is majorly caused by oxidative damage to these proteins.

Actually, the hepatocytes can be described as favorite targets of oxygen species attacks. In the hepatocyte itself, mitochondria could be the first step of the cell damage. Affected mitochondria suffers from corruption of the internal system, low activity in enzymatic action and metabolism process leading to intrinsic cell dysfunction (Ramadan et al., 2001; Yilmaz et al., 2005).

Tempol is a member of nitroxides compound which is able to accelerate the metabolism of reactive oxygen species (ROS) in liver and preserve hepatic mitochondria from oxidative damage and promote tissues oxygenation. Morever, tempol reduces hepatic steatosis, fibrosis and has activity against alcoholic and non-alcoholic liver diseases (Wei et al., 2009).

According to Kang (2004), Silymarin has hepatoprotection and anticarcinogenic activity. This fact relied to the presence of hydroxyl group at the C5 and carbonyl at C4 which increases the capability of silymarin to chelate with ferrous

ions, synergize the scavenging to the maximum, and potentiate the lipid peroxidation inhibition by binding with peroxy radicals (Abu ghadeer et al., 2001). All of these mechanisms result in significant increment in the defense machinery.

In our study, there is an agreement with previous studies that has shown the contribution of oxidative stress, lipid peroxidation and mitochondrial impairment in cisplatin-induced hepatotoxicity (Ramadan et al., 2001; Yılmaz et al., 2005).

As a conuclusion, this study proves the hepatoprotective actions of tempol and silymarin against cisplatin induced hepatotoxicity.this protection effect was confirmed by measuring malonaldehyde (MDA), total anti-oxidant activity (TAS), myeloperoxidase (MPO) and by histopatholigical examination.

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Web sites:

(www.online.lexi.com updated 16/12/2015).

9. ATTACHMENTS

Attachment 1. Experimental Animal Ethic Committee Approval

MARMARA ÜNİVERSİTESİ HAYVAN DENEYLERİ YEREL ETİK KURULU
PROJE ONAY FORMU

BAŞVURU BİLGİLERİ	PROTOKOL KODU	56.2014.mar	Çalışma: Yükseklisans
	PROJE ADI	Sıçanlarda Tempolün ve Silymarin Sisplatin Kaynaklı Hepatotoksisite Üzerine Etkilerinin İncelenmesi	
	SORUMLU ARAŞTIRICI ÜNVANI/ADI	Prof.Dr. Fikret Vehbi İZZETTİN	
	ARAŞTIRMA MERKEZİ	Marmara Üniversitesi Eczacılık Fak. Klinik Eczacılık ABD LAb.- Marmara Üniv. Deneysel Araştırma ve Hayvan Lab.	
	DESTEKLEYİCİ	BAPKO	

KARAR BİLGİLERİ	Tarih 02.10.2014 Yukarıda başvuru bilgileri verilen araştırma başvuru dosyası ve ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş ve gerçekleştirilmesinde sakınca bulunmadığı için Kurulumuzca onaylanmasına oy birliği ile karar verilmiştir. Onay sonrasında yapılacak her türlü proje değişiklikleri (katılımcılar, başlık vb.) veya protokol değişikliklerinin Etik Kurula bildirilerek proje onayının yenilenmesi gerekmektedir.
ETİK KURUL BİLGİLERİ	
ÇALIŞMA ESASI	Deney hayvanları ile yapılacak olan bilimsel araştırma, test, sağlık hizmetleri uygulamaları ve eğitim-öğretim gibi temel etkinliklerde kullanılan yöntem ve materyaller ile ilgili etik standartları gözetmek, etik ilkeler doğrultusunda görüş bildirmek, araştırma önerilerini incelemek ve sertifikası olmayanların deney hayvanı kullanmalarını engellemektir.

ÜYELER

Unvanı / Adı / Soyadı	Uzmanlık Dalı	Kurumu / EK Üyeliği	Onaylanan Proje ile İlişkisi	Toplantıya katılım	İmza
Prof. Dr. Göksel ŞENER	Farmakoloji	M.Ü Tıp Fakültesi ve Deney Hayvanları Uygulama ve Araştırma Merkezi Müdürü	Var Yok	Evet Hayır	
Prof.Dr. İnci ALİCAN	Fizyoloji	Yürütücü Sekreteri	Var Yok	Evet Hayır	
Prof. Dr. Ayşen YARAT	Biyokimya	M.Ü Diş Hekimliği Fakültesi	Var Yok	Evet Hayır	
Doç.Dr. Serap ŞİRVANCI	Histoloji Embriyoloji ABD	M.Ü Tıp Fakültesi	Var Yok	Evet Hayır	
Doç.Dr. Hasan Raci YANANLI	Farmakoloji	M.Ü Tıp Fakültesi	Var Yok	EVET HAYIR	
Doç.Dr. Gürkan SERT	Tıp Tarihi ve Etik	M.Ü Tıp Fakültesi	Var Yok	Evet Hayır	
Vet. Hek. Dilek ÖZBEYLİ	Veteriner Hekim	M.Ü Tıp Fakültesi ve Deney Hayvanları Uygulama ve Araştırma Merkezi Sorumlu Veterineri	Var Yok	Evet Hayır	
Bio. Arif GÜMÜŞ	Biyoloji	İstanbul Hıfzıssıhha Enstitüsü Müdürlüğü, Kurumla ilişkisi olmayan TC vatandaşı üye	Var Yok	Evet Hayır	
Billur AYGÖR	Emekli Memur	Kurumla ilişkisi olmayan TC vatandaşı üye	Var Yok	Evet Hayır	

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Protective Effects of Tempol and Silymarin Against Cisplatin-Induced Hepatotoxicity In Rats

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This study aims to evaluate the protective effects of tempol and silymarin against cisplatin induced hepatotoxicity in rats.

Rats were divided into six groups consisting of six animals: three control groups, one toxicity group, and two treatment groups. Serum saline (1ml/kg), and silymarin (100mg/kg) were administrated by oral gavage while tempol (15mg/kg) administrated by intraperitoneal injection for seven days. Single dose of cisplatin was given on the seventh day of the experiment.Livers were removed after 24 hours of cisplatin administration within each group. Malondialdehyde (MDA) levels and total antioxidants status (TAS) were measured in rats' hepatic tissues and a histopathological examination of hepatic tissues was also done.

Tempol and silymarin significantly decreased liver MDA levels, which had increased as a result of cisplatin administration ($p<0.05$). TAS level in cisplatin toxicity was significantly decreased when compared with control groups; besides The TAS level in silymarin and tempol treatment group was significantly elevated when comparison with cisplatin toxicity group ($p<0.05$). These results were concordant with histopathological examination.

Tempol and silymarin found to be effective in preventing cisplatin induced hepatotoxicity.

10. CURRICULUM VITAE

Name	Tayf	**Surname**	Alqozbakr
Birth Place	Mosul-Iraq	**Birth Date**	06.01.1985
Nationality	Iraqi	**E-mail**	anmartaif@yahoo.com

Education

	Name of Graduation Foundation	**Graduation Year**
Master Degree	Marmara University Clinical Pharmacy Department	2015
University	Mosul University Faculty of Pharmacy	2007
High School	Al-mutamayzeen-Ninevah Secondary School	2002

Working Experience

Job	**Company**	**Duration (Year - Year)**
Pharmacist	Ibn Sina Teaching Hospital	2008-2009
Pharmacist	University of Mosul-College of Pharmacy	2009-2012
1 Pharmacist	Various Pharmacies	2007-2012

Foreign Languages	**Reading***	**Speaking***	**Writing***
English	Very Good	Very Good	Very Good
Arabic	Very Good	Very Good	Very Good
Turkish	Very Good	Very Good	Very Good

Foreign Language Exam Evaluation#

YDS	ÜDS	IELTS	TOEFL IBT	TOEFL PBT	TOEFL CBT	FCE	CAE	CPE
				497				

Computer Knowledge

Program	**Usage Skill**
Microsoft office	Very Good

***** Please estimate as very good, good, medium, weak.