FORMULATION DEVELOPMENT OF SILYMARIN NANOPARTICLES, AND EVALUATION OF HEPATOPROTECTIVE EFFECT

by

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FORMULATION DEVELOPMENT OF SILYMARIN NANOPARTICLES, AND EVALUATION OF HEPATOPROTECTIVE EFFECT

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ABSTRACT

FORMULATION DEVELOPMENT OF SILYMARIN NANOPARTICLES, AND EVALUATION OF HEPATOPROTECTIVE EFFECT

Last decades, many individuals have preferred some medicinal plants to treat the diseases they suffer from and to prevent various diseases. Therefore, recent scientific studies have focused on investigating the effects of plants on diseases. The *Silybum marianum*, used onwards first ages and whose effects on the liver are known, is one of them. In this study, the effects of *Silybum marianum* active substances silymarin, and their nanoparticles on the liver disease were observed. While the nanoparticles were prepared using soy lecithin, *Silybum marianum* extracts, which are active ingredients with the incorporation technique, were obtained from three different sources. In the light of previous studies, it has been seen that it is appropriate to mix soy lecithin and extracts in a 1:1 ratio. While soy lecithin is being prepared, the homogenizer and probe sonicator technique are applied, while *Silybum marianum* extracts are prepared by solvent extraction method. After incorporation, the probe sonication method was used to mix, decrease the particle size and be a homogenous mixture. Moreover, in order to increase the stability, the freeze-dried technique was used for soy lecithin, extracts, and the final formulations. The characterization of formulation size, zeta potential, and PDI was observed by using the DLS technique, the release profile of three different formulations, and prepared calibration curve were performed by using HPLC. *In vivo* studies, the procedures were performed on four different groups which were determined as the nanosilymarin formulation applied after the liver damage was induced, the other was treated with pure silymarin formulation under the same condition, the control group (healthy) and the last one who did not receive any treatment after induced liver damage. All groups have six Sprague Dawley, male and young rats. After that, histopathological and HPTLC tests were done. As a result of *in vitro, in vivo* animal experiments and histopathological studies, it has been observed that nanoparticle-containing standardized seeds are more effective.

ÖZET

SİLYMARİN İÇEREN NANOPARTİKÜL FORMÜLASYONU GELİŞTİRME VE HEPATOPROTEKTİF ETKİSİNİN DEĞERLENDİRİLMESİ

Son yıllarda, çoğu insan muzdarip olduğu hastalıklarını tedavi etmek ve bazı hastalıklardan korunmak için şifalı bitkileri tercih etmektedir. Bu yüzden, son bilmsel çalışmalar bitkileri hastalıklar üzerine etkisini araştırma üzerine yoğunlaşmıştır. İlk çağlardan beri kullanılan ve karaciğer üzerine etkisi bilinen *Silybum marianum* bunlardan bir tanesidir. Bu çalışmada, *Silybum marianum'un* aktif maddesi olan silymarin ve bunun nanopartiküllerinin karaciğer hastalıkları üzerine etkisi gözlemlenmiştir. Soy lesitin kullanarak nanopartiküller hazırlanırken, incorpore yönetmiyle içlerine koyulacak aktif madde *Silybum marianum* ekstraktları üç farklı kaynaktan elde edilmiştir. Daha önce yapılan çalışmalar ışığında, soy lesitin ve *Silybum marianum* ekstraktarının 1:1 oranında karıştırılmasının uygun olduğu görülmüştür. Soy lesitin hazırlanırken homojinazatör ve prop sonikatör tekniği uygulanırken silymarin ekstraktlarıda çözücü ekstraksyon yöntemiyle hazırlanır. İnkorporasyon yapıldıktan sonra karıştırmak, partikül boyutunu küçültmek ve homojen karışım olması için prop sonikatör kullanılmştır. Ek olarak stabiliteyi arttırmak için soy lesitine, ekstraktlara ve son formülasyonlara dondurarak kurutma yöntemi kullanılmıştır. Formülasyonun karakerizasyonu, boyutu, zeta potansiyeli ve PDI DLS yöntemiyle gözlenmiştir, üç formülasyonunda dağılma profili ve kalibrasyon grafiğide HPLC kullanarak yapılmıştır. *İn vivo* çalışmalarda, prosedür dört farklı gruba uygulanmıştır ve grupların bir tanesi karaciğer hasarı oluştuktan sonra nanopartikul uygulanan, diğeri aynı koşullarda saf silymarin ile tedavi edilen, kontrol grubu (sağlıklı) ve son olarak karaciğer hasarı olduktan sonra hiçbir tedavi almayan grup olarak belirlenmiştir. Bütün gruplarda altı tane Spraque Dawley, erkek ve genç sıçan vardır. Bundan sonra histopatolojik çalışma ve HPTLC testleri yapılmıştır. *İn vitro, in vivo* hayvan deneyleri ve histopatolojik çalışmalar sonucu standardize tohum ekstraktı içeren nanopartikülün daha etkili olduğu görülmüştür.

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LIST OF SYMBOLS/ABBREVIATIONS

A1AD	Alpha-1-antitrypsin deficiency
ACE	Angiotensin Converting Enzyme
ALP	Alanine phosphatase
ALT	Alanine aminotransferase
APAP	Acetominophen
AST	Aspartate aminotransferase
ATP	Adenintriphosphate
BSEP	Bile salts export pump
COOH	Carboxyl
CYP2C8	Cytochrome P450 Family 2 Subfamily C Member isoenzyme
CYP2C8	Cytochrome P450 Family 2 Subfamily C Member isoenzyme
Cys-34	Cystenilated
DNA	Deoxyribo nucleic acid
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GGTP	Gamma-glutamyl transpeptidase
Glucose-1-P	Glucose 1 phosphate
Glucose-6-P	Glucose 6 phosphate
GOT1	Glutamic oxaloacetictransaminase 1
GOT2	Glutamic oxaloacetic transaminase 2

GSH	Glutathione
GSTs	Glutathione S-transferases
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
HSCs	Hepatic stellate cells
LDH	Lactate dehydrogenase
LSECs	Liver sinusoidal endothelial cells
NAC	N-acetyl cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NaOH	Sodium hydroxide
NAPQI	N-acetyl-p-benzoquinone imine
NH2	Amino
Nm	Nanometer
NP	Polyethylene glycol
OATP	Organic anion uptake transporter peptides
OH	Hydroxyl
P5P	Pyridoxal-5-phosphate
PBC	Primary biliary cirrhosis
PDI	Poly dispersity index
P-gp	P-glycoprotein
PSC	Primary sclerosing cholangitis

PSIL	Pure silymarin
R^2	Correlation coefficient
RNA	Ribo nucleic acid
Rpm	Rotation per minute
SD	Standard deviation
SH	sulfhydryl
SL:PSIL	Pure silymarin in soy lecithin nanoparticle
SL:SSE	Standardized silymarin seed extract in soy lecithin nanoparticle
SL:LME	Local market silymarin seed extract in soy lecithin nanoparticle
SSE	Standardized seed extract
TLRs	Toll-like receptors
LME	Local market seed extract
TNF-α-	Tumor necrosis factor
TPO	Thrombopoietin
UDP	Uridine diphosphate
USP	United states pharmacopeia
UV	Ultraviolet
V	Volume
w/v	Weight per volume

1. INTRODUCTION

In recent years, millions of people are suffering from hepatic illnesses in the world. The rate of mortality because of major liver illnesses such as acute hepatitis, cirrhosis, and liver cancer is increasing day by day [1]. In 2017, liver diseases are the eleventh cause of death, but in spite development of vaccines and antiviral actives in recent years, approximately four percent of death worldwide are caused by liver diseases [1,2]. Despite the moral values, some of the active materials included in the plants are very effective for vital diseases. Silymarin acquires from *Silybum marianum* has been proven to be useful in the therapy of liver diseases [3].

Silymarin is a flavonoid that is extracted from the *Silybum marianum* or Milk thistle is as an English name. It contains flavonolignans from 65-80 percent and these are Isosilybin A and B, Silydianin, Silybin A and B, and Silychristin. In addition, it includes with a trace of fatty acids and polyphenolic compounds [4,5].

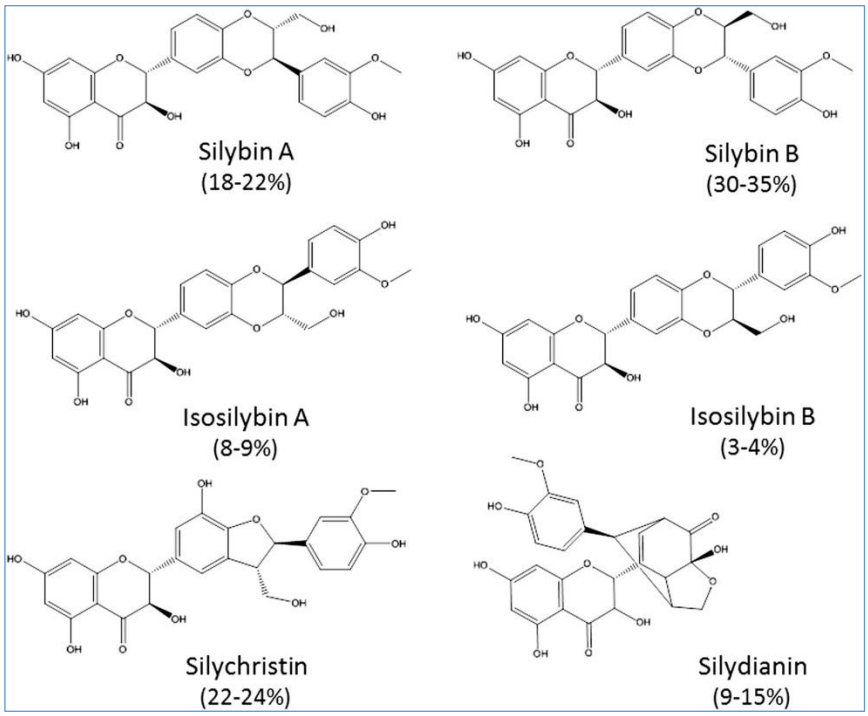

Figure 1.1. Components of silymarin mixture [6]

Silybum marianum is a biannual plant and has purple flowers. Although they grow at 3 meters high, 1 meter wide, and in warm and dry soil climates, they are commonly in sizes between 0.9 and 1.8 meters. The main countries are Southern Europe and Russia, Asia, Northern Africa [7].

Silybum marianum or its extract or seeds have been used from ancient times for many disorders and their therapies [3]. It prevents cancer, pancreas, hemolysis, against environmental toxins, against mushroom poisoning, or their therapies. In addition, it is used for antiosteoporotic, renal protection, neuronal effects, immune modulation and is applied as a selective estrogen receptor modulator. Lastly, it is used on liver disorders it is the most important effect of silymarin [8].

While the steroid structure of silymarin increases DNA and RNA synthesis, the regenerative ability of liver cells increases in the same way. Additionally, modification of hepatocytes outer membrane decreases the entry of some microorganisms as xenobiotics into the cell and these are been used in mushroom poisoning and liver diseases. In recent years, the effect of silymarin on liver cancer and disease has been investigated. In the study, the low bioavailability problem is an attempt to overcome by the different new technologies [8,9].

2. THEORETICAL BACKGROUND

2.1. NANOTECHNOLOGY

In last years, the nanotechnology term has been very popular all over the world in the scientific fields. Although this term is defined differently by many individuals and institutions, its essential content always emphasizes the same thing: particles in the nanometer size. It was defined as, study of structures from 1 to 100 nanometers (nm) in size. The broad definition of nanotechnology from European Commission "is the understanding and control of matter at dimensions between approximately 1 and 100 nanometers, where unique study enable novel applications and it is encompassing nanoscale science, engineering, and technology, nanotechnology involves imaging, measuring, modeling, and manipulating matter at this length scale" [10].

2.1.1. History of Nanotechnology

The wonder of humans increased by their power of imagination. They try every way to make their dreams come real. In twenty-first century, nanotechnology was born out of the dream of Richard Zsigmondy. He was first recommend the nanometer term [11]. The father of modern nanotechnology was Richard Feynman. He was started with one hypothesis," There's plenty room at the bottom" and there after, new ideas and findings were evidence of this hypothesis [12].

2.1.1. Fields of Nanotechnology

The novel nanotechnology has a wide range of working fields. Its useful features and advantages made it popular in all areas of scientific study.

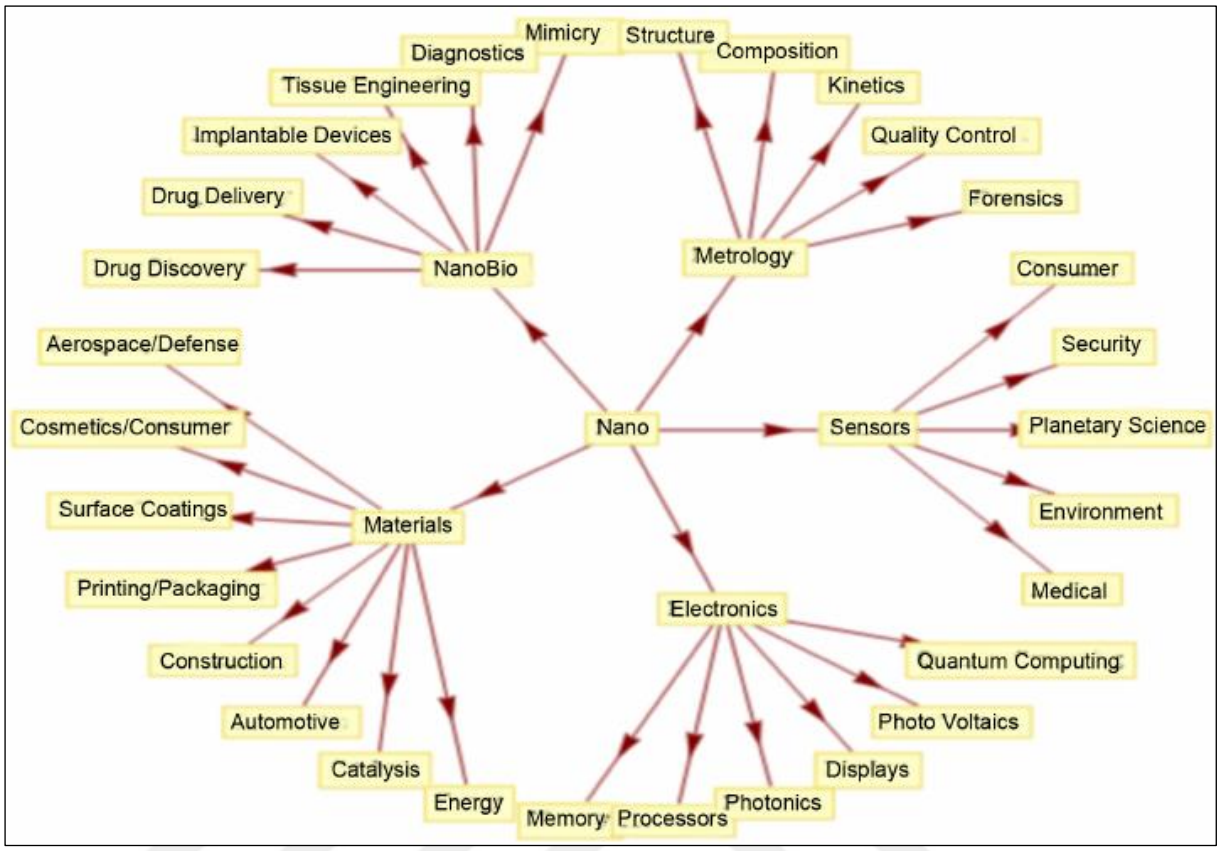

Figure 2.1. Fields of nanotechnology [13]

The most popular area is nano-biotechnology studied on the health of individuals. They develop the drug innovations and drug delivery systems such as novel drugs to decrease the side effects of drugs. For drug delivery systems of the nanotechnology provides more benefits such as

- Improve delivery poorly water soluble drugs
- Provide targeting
- Drug stays for a long time and shows more efficiency in therapy
- Bioactivation of drug is extended
- Movement of drugs between endothelial and epithelial membranes
- Using both diagnostic and therapeutic modalities together with using one active [14].

The main goal of nanotechnology is to produce nano-size particles called nanoparticle. The novel drugs, drug delivery systems included the nanoparticle.

2.2. SILYBUM MARIANUM

Silybum marianum (S. marianum), which belongs to family, was an annual plant native to the Mediterranean region. Additionally, it has grown up in a warm and dry area. The name was *Silybum marianum* obtains seeds and active material was a complex, silymarin. This material was used in a wide range of areas such as treatment of liver diseases or hepatoprotection effect, protection against breast cancer [15], ovarian cancer, lung cancer [16], liver carcinoma [17], and colon cancer [18], renal and pancreatic protection, neuronal effect, immunomodulation protect against hemolysis and environmental toxin [8]. In addition, it is used for mushroom poisoning, antioxidant effects, anti-inflammatory, modifying cell transporters. On the other hand, the working principle and pathways of the silymarin are not clear yet. The four known mechanisms are shown in Figure 2.2.

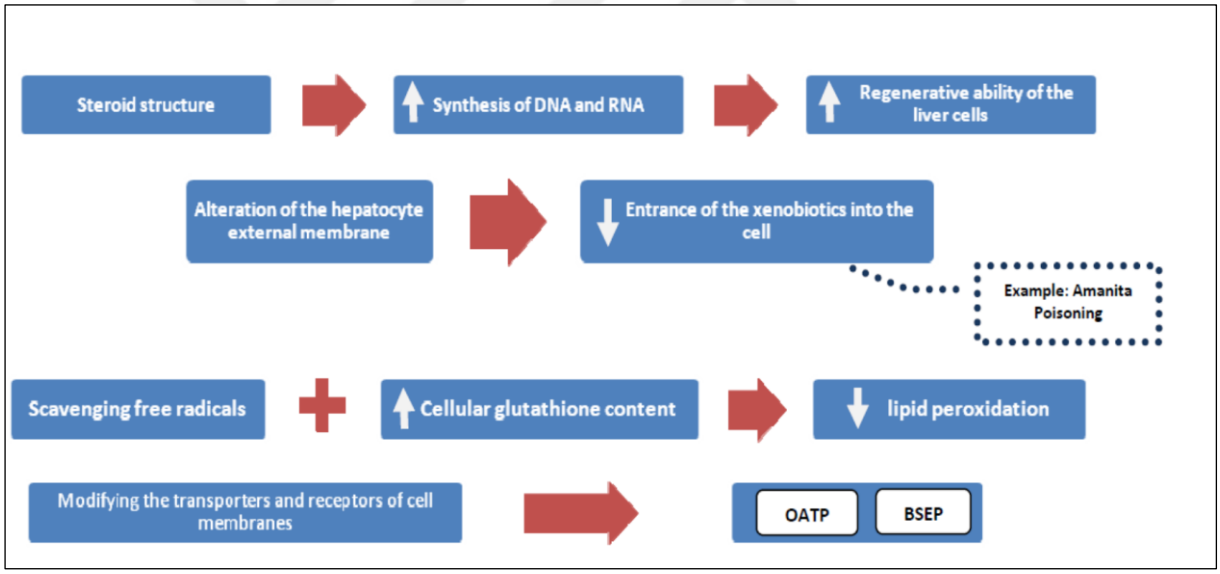

Figure 2.2. Different mechanisms of action of silymarin [19,20]

The first concern of about the structure of silymarin. Similar to the structure of liver cells, the steroid property of silymarin increases the regenerative capacity of hepatic cells by increasing the synthesis of the largest gene storage materials called DNA and RNA. The second one was modifying the structure of the hepatocyte's outer membrane that creates the barrier to entry of the xenobiotics into the cell-like Amanita mushroom poisoning. The other one was increasing glutathione content with scavenging free radicals which inhibit the lipid peroxidation. The last one was altering the transporters and receptors of cell membranes like

organic anion uptake transporter peptides (OATP), bile salts export pump (BSEP), ABC transporters (P-gp), and TNF-α-dependent transporters [19,20].

2.2.1. Pharmacokinetic Property of Silymarin

2.2.1.1. Absorption of silymarin

Silymarin is very low soluble in water so it was used orally as a tablet or capsule including its extracts. After the oral application, the absorption level was low. In the studies with rats after 24 hours of oral application of silymarin was indicated ratio as 2-3 percent in the bile. For humans, the maximum plasma concentration arrives after 4-6 hours following oral intake [21].

2.2.1.2. Metabolism of silymarin

After oral intake, silymarin is exposed to both phases I and II metabolisms [22]. The main metabolite of silymarin is O-demethylated end products that are produced by CYP2C8 [23]. Additionally, there are monohydroxy and dihydroxy metabolites [24]. The silymarin is exposed to fast and excessive phase II metabolism and it leads to low bioavailability. However, fifty-six individuals diagnosed with non-alcoholic fatty liver disease (NAFLD), mutant allele UGT1A128 produced little differences inter-subject in silymarin pharmacokinetics [25].

2.2.1.3. Elimination of silymarin

Silymarin is conjugated with sulfate and glucuronic acid in the liver and is eliminated with bile. When it arrives in the bowel, it is hydrolyzed with the effect of intestinal flora and absorbed again. In some studies, it has been reported that the maximum absorption rate in oral intake reaches 35 percent [26]. The half-life of silymarin was 6-8 hours. They are generally eliminated through bile or urine on a low amount [27].

2.3. LIVER

The liver is one of the most vital organs for humans due to play a major role in metabolism and the functions including excretion of waste metabolites and xenobiotics, protection against foreign substances, detoxifying toxic side effects of drugs, and secreting bile to metabolize the lipid. Additionally, it was involved in protein synthesis, production of digestion enzymes, and synthesis as well as a breakdown of small or complexes [28].

2.3.1. Structure of Liver

As is known, the liver is a crucial part of the body and regulates metabolic effects. The liver is located in the upper right-hand part of the abdomen, under the diaphragm, top of the stomach, right side of kidney and intestines, overlies gallbladder of the healthy individuals [29,30]. It is a wedge-shaped, reddish-brown, approximately 1.5 kg weight, 15 cm in length, and has two lobes consisting of eight segments [31,32].

The functions of the liver are regulated by the two lobes. Both lobes have eight segments have of 1000 small lobes, lobules. They are built up with millions of hepatic cells are called hepatocytes [33]. The lobules are joined together with a little channel called ducts which bind to larger ducts that form the common hepatic duct. The bile is secreted by the liver, was send to the gallbladder and small part of the intestines duodenum.

In addition, the liver is a blood supplier to the body. The thirteen percentage of blood is moved by the blood vessels to the liver. One of them is the hepatic artery that sends rich oxygen blood, the second one is the hepatic portal vein which sends nutrient-rich blood to the liver [29].

2.3.2. Functions of Liver

The liver has plenty of functions such as bile secretion, biotransformation of bilirubin, hematological and vascular functions, biotransformation of nutrients, detoxification,

minerals and vitamins storage, endocrinal, immunological protective functions, and inactivation of toxins.

2.3.2.1. Bile secretion

The liver is secreted 700-1200 ml of bile/day for intestinal digestion. Bile is a yellowish-green fluid, alkaline, bitter-tasting includes bile salts, cholesterol, electrolytes, bilirubin (a pigment), and water. It is produced by hepatic cells and secreted into the canaliculi. Salts of bile function is metabolites and digest the fats. Fat emulsification and absorption process as flows.

- Acid-dependent fraction of bile is secreted by hepatocytes includes bile salts, cholesterol, phospholipids, and bilirubin. Similarly, bile secreted by hepatocytes is secreted from epithelial cells of the bile canaliculi. A bicarbonate-rich aqueous fluid requires alkaline pH.
- The primary and secondary bile acids are produced by conjugation process. The primary bile acids are synthesized by hepatocytes using cholesterol. However, the secondary bile acids are produced by the action of intestinal bacteria in the small intestine, after that, these bacteria are absorbed and enter into the liver.
- Bile salts occur by conjugating amino acids with bile acids.
- Conjugation process of bile salt provides more solubility in water to the bile acids, thus limiting diffusion rate of bile acids from the intestines [34].

2.3.2.2. Biotransformation of bilirubin

Bilirubin is a potentially toxic material that regulates the complex that serves regulation of iron to some proteins. Additionally, it is a vital metabolite of liver and it is been made nontoxic by the detoxification and disposition process of liver. Bilirubin is been produced by breaking down of hemoglobin and destroying the erythrocyte cells [35,36]. The metabolism of bilirubin in the liver is been happened in two steps, albumin binding and hepatic transport mechanism (Figure 2.3).

- The first step of albumin binding begins when bilirubin reaches the plasma and the transporter in the body thanks to albumin. Absence of unconjugated bilirubin (non-albumin bound) which are located in plasma, binding affinity is very high with the optimal condition. The more permeable hepatic circulation process lets albumin bilirubin complex structure to reach the sinusoidal surface of the liver cells,

indicating that this complex reaches the liver. After the dissociated pigment is been entered, the liver is relatively ineffectual with the first pass clearance of bilirubin. As a result, unconjugated bilirubin concentration bound to albumin in the venous circulation can be calculated. This all these processes are reversible.

- Hepatic transform mechanisms have two different ways. One of these is passive diffusion and it occurs according to the concentration gradient appropriate with first degree kinetic. The second one is receptor-mediated endocytosis is used carrier proteins. The entrance in the hepatocytes of unconjugated bilirubin happens with extraction in the periportal regions. A part of all bilirubin that is conjugated or unconjugated is moved backward of the sinusoidal space and this part is taken up downstream to the sinusoidal flow again. This process is happened by the organic anion transporting polypeptide (OATP). Conjugated bilirubin is excreted in the urine and it escapes reuptake into the liver cells. After bilirubin binding to glutathione S transferases increases net uptake and to make minimizes internalized bilirubin efflux [37].

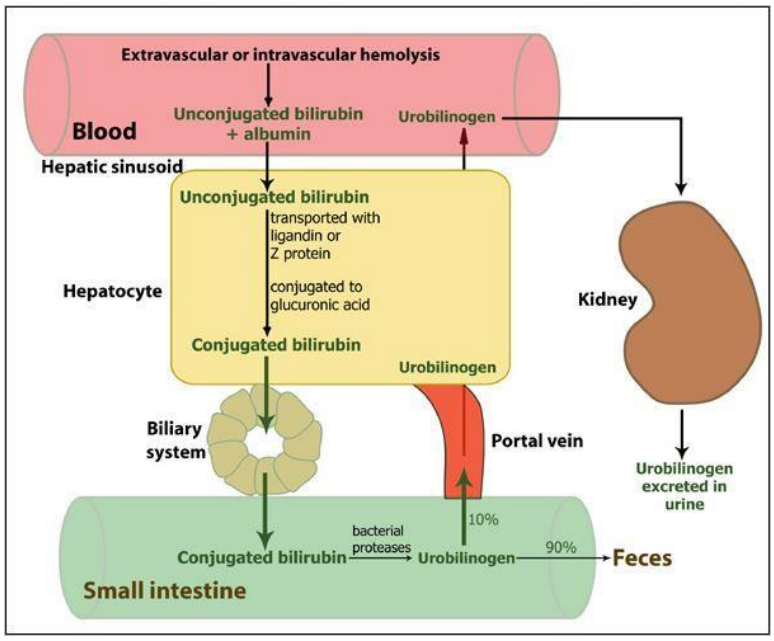

Figure2.3. Metabolism in bilirubin [38]

2.3.2.3. Hematological and vascular functions

Blood is a vital fluid for humans. Transportation nutrients, feelings also hormones are controlled by the blood. The liver receives 30 percent of resting cardiac output so, it can be accepted the vascular organs in our body. This hepatic vascular system has release and store ability for the vital fluid and it is a reservoir for the general circulation, which shows that it is a dynamic system. Generally, the liver has 10-15 percent of total blood and more of these located in the sinusoids. When the blood level is been decreased the liver adjusts its blood volume and replaces the blood for the amount of hemorrhage. In contrast, the way that, volume has increased the fluids are infused, the hepatic blood volume expands and adjusts systemic circulation blood volume [39].

2.3.2.4. Biotransformation of nutrients

The liver satisfies the small molecules of the three major digestible polymers such as glucose, amino acids, fatty acids, and glycerol. The liver is used as a storage for these molecules if they are necessary, it requires them [40] for the carbohydrates in the liver play an important role because of demanding energy from glucose. According to the needs of the human body, the liver synthesizes, stores, and oxidizes the glucose. These processes are been arranged by covalent modification, allosteric mechanism, transcriptional factors, substrate availability, and hormones [41]. The critical aspects of the transformation in liver are essential for the conversion and provide the energy.

- The liver oxidized the triglycerides to provide some energy
- The major part of lipoproteins are been produced in the liver
- Carbohydrates are been converted to proteins, fatty acids and triglycerides give and store in adipose tissue.
- The liver plays a key role to synthesize a large amount of cholesterol and phospholipids.

Some of them stored at lipoproteins and it is used to the rest of the body. If it is available for the body, it is excreted bile as cholesterol after conversion to bile acids.

The transportation of protein is a crucial because they are vital molecules [42].

- Amino acids' deamination and transamination and glucose or lipids are produced by conversion of the non-nitrogenous part of those molecules.

- One of the aims of synthesizing urea is to remove toxic ammonia from the body and so prevention central nervous system disease.
- The liver synthesizes non-essential amino acids [43,42].
- Numerous of the plasma proteins production is responsible for the hepatocytes. Albumin is the main plasma protein, is been produced by the liver. In addition, the liver plays a role in blood coagulation while producing the clotting factors [44].

2.3.2.5. Detoxifications

The liver has a crucial role in protecting the organism against toxins and harmful metabolites are been produced by itself. Some lipophilic materials are been converted to the substance of soluble and are eliminated with urine from the body. This protective property of liver cells comes from having lots of xenobiotic-metabolizing enzymes. Their common function is catalyzation of the vital and/or phase reactions of the liver. They affect functional groups of drug and chemical molecules [45].

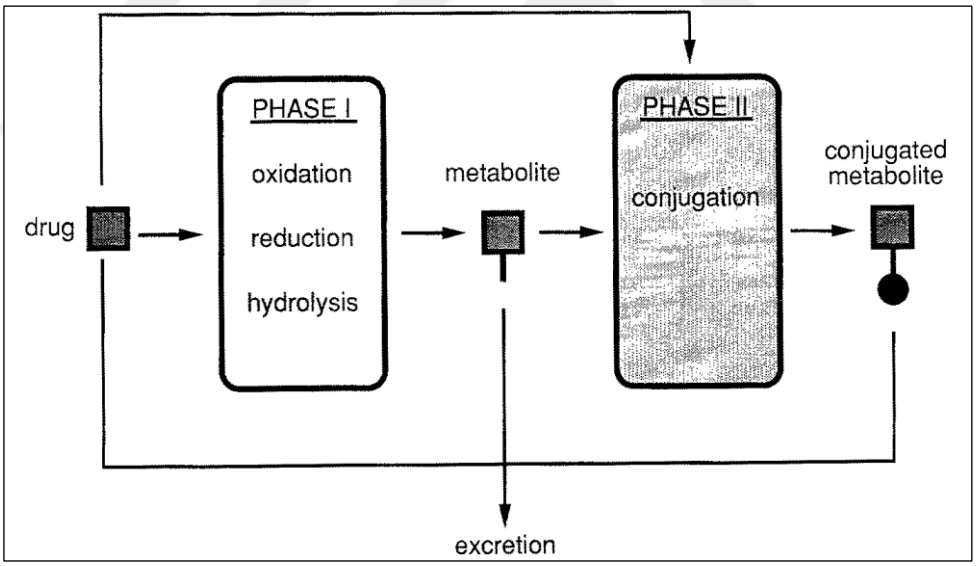

Figure 2.4. Phase I, II reaction [46]

Phase I reactions are performed by many enzyme classes and they can add or expose functional groups (often hydroxyl groups) to the drug molecule. In this way, the excretion of the molecule becomes easier. The molecule allows reacting with the enzyme of Phase II reactions. These are conjugation or synthesis reactions. These reactions aim to enhance excitability by adding water-soluble side groups. The biotransforming certain xenobiotics

such as xanthine dehydrogenase, catechol O-methyltransferase ability of enzymes. Furthermore, to perform the intermediary metabolism of endogenous substrates. Although several enzymes like arylamine N-acetyltransferase has no known endogenous substrates, they have evolved specifically for the aim of preventing environmental chemical inputs [47].

2.3.2.6. Minerals and vitamins storage

The liver depots A, D, E, K, and B12 vitamins, nicotinic acid, and folic acid [48].

- Vitamin A is responsible for the vision and immune functions, reproduction, and cellular communication. Vitamin A plays a role in the heart and kidney's function, too [49,50].
- Vitamin D is been produced in the body itself as cholecalciferol form or taken from food as ergocalciferol. Vitamin D could be activated by metabolization in the liver. Its functions are to adjust the concentration of calcium and phosphate in the serum of blood, enhance calcium absorption is located in the kidneys and intestines, improve mobilization of calcium from parts of the skeletal system.
- Vitamin E is stored in the liver or adipose tissue. It is used as an antioxidant and is responsible for preventing propagation of free radicals of protection vitamin A.
- Vitamin K is obtained from animal and plant products. Vitamin K functions are the synthesis of clotting factors II, VII, IX, and X and be co-factor for enzymes.
- Vitamin B12, cobalamin, is certainly obtained from an animal source. Approximately, 50% of the total amount of Vitamin B12 is placed in Vitamin B12 is responsible for the production of DNA, RNA, and red blood cell and carrying on healthy neurons
- Iron levels need to be regulated so the liver stored the excess amount of iron. Although iron is depoted on the ferritin protein produced by the liver, the big amount is stored in hepatocytes. More excess iron is stored in haemosiderin, the large and insoluble complex is not mobilized effectively.
- Copper is a key to activate the enzymes, regulating energy production and it also placed iron metabolism and brain function.
- The liver maintains the blood glucose level. After taking nutrients, the glucose level was increased and the liver or skeletal muscle store glucose as a polymer called glycogen. Glycogen synthesis of the process is performed four crucial steps. The first step of the synthesis of glycogen is converting from glucose to glucose-6- phosphate by glucokinase enzyme for liver or hexokinase enzyme for skeletal muscle. The

second step is a conversion from the first step production glucose-6-P to glucose-1-P by phosphoglucomutase then glucose-1-P is been converted to UDP-glucose. The last step is UDP-glucose is been added to the glycogen chain or glycogen synthesis or enzymes [51].

2.3.2.7. Endocrine functions

The liver secreted important hormones are called angiotensinogen, thrombopoietin, hepcidin, and betatrophin.

- Antinotensionegen is a protein, released into the blood and used as a precursor for angiotensin. This one is secreted by the liver but the kidney manufactures the renin. One of the important functions of the kidney is to observe the blood pressure if it is required it is corrected and secreted the protease renin. Renin affected angiotensinogen and produced angiotensin I. Angiotensin I is been joined by Angiotensin Converting Enzyme (ACE) and to produce angiotensin II. Its function is to constrict walls of arterioles closing down capillary beds, to stimulate the proximal tubules and adrenal cortex to release aldosterone, enhanced heartbeat strength, and stimulate the pituitary in order to release the vasopressin.

- Thrombopoietin (TPO) is a protein, activates precursor cells in the bone marrow to differ into megakaryocytes. It is been used in the hemostasis of individuals.

- Hepcidin is a peptide, inhibits the release of iron from intracellular stores in the body. Thus hepcidin is known as an antimicrobial peptide and part of the important immune system.

- Betatrophin is a protein that provides insulin secretion from beta cells found in the Langerhans islets of the pancreas. Betatrophin injections are used by recombinant DNA technology and it may be a useful treatment for diabetes mellitus [52].

2.3.2.8. Immunological protective functions

Immune responses are forced by parenchymal cells, hepatocytes, and cholangiocytes, hepatic stellate cells (HSCs), and liver sinusoidal endothelial cells (LSECs) which are located between nonparenchymal cells, also act as the first sensors [53]. Toll-like receptors (TLRs), classic danger-sensing receptors, activate the immune system, are defined on hepatocytes [54], LSECs, and HSCs [55]. The immunological protective functions of the liver are described.

- Myeloid and lymphoid cells are immune cells that were placed in the liver. Therefore, the liver plays an important role in the immune system.

- The gastrointestinal tract and gut-derived endotoxins have antigen-rich blood that enters the sinusoids. Suppression of innate and adaptive immune responses are performed by some mechanisms of hemostasis and the result of this is tolerance.

- Under conditions in liver diseases, mechanisms of Toll-like receptor signaling or inflammasome activation and molecular danger patterns (alarmins) initiate inflammatory responses, whose results are the chemokine-mediated hepatic infiltration of circulating leukocytes, should be conserved.

- Kupffer cells and macrophages transcribe their phenotype satisfy in perpetuating inflammation and wound healing, local signals and apply important functions. However, it may also play a role in the healing of fibrosis and inflammation.

- Innate lymphocytes are another source of immunoregulatory cytokines in diseased livers and contribute to eliminating activated myofibroblasts and infected, injured, or malignant cells.

- Hepatic T cells are activated as a result of damage and result in direct cytopathic functions as well as immunostimulating cytokine secretion, especially in viral and autoimmune hepatitis [56].

2.3.2.9. Inactivation of toxins and drugs

Toxins and drugs are inhibited by reactions series performed in the liver. They have two major steps, phase I and phase II.

- Phase I reactions

These are called also nonsynthetic reactions include hydrolysis, oxidation, reduction, decyclization, and cyclization. These oxidative reactions include cytochrome P450 monooxygenase (CYP), NADPH, and oxygen. For phenothiazines, paracetamol, and steroids this method is preferred to their metabolism. When the metabolites become polar enough, they can be easily eliminated. Oxidation reaction involves some systems which are called, alcohol dehydrogenase, cytochrome P450 monooxygenase system, aldehyde dehydrogenase, flavin-containing monooxygenase system, and monoamine

oxidase, and co-oxidation by peroxidases. The reduction has two major enzymes. One of them is NADPH-cytochrome P450 reductase. This is a membrane-bound enzyme that entailed electron movement to cytochrome in the eukaryotic cell. The general electron flow system followed from enzyme to oxygen.

$$NADPH \rightarrow FAD \rightarrow FMN \rightarrow P450 \rightarrow O2$$

The second one is Reduced (ferrous) cytochrome P450 which joins the futile cycling in order to take free radical electrons and lose oxygen. As a result of these reactions superoxide anion form is produced. The last major step is hydrolysis which has two enzymes, esterases, and amidase, epoxide hydrolase. This is separating step is metabolite as breakdown the bonding of the molecules and drugs.

- Phase II

In phase II reactions, xenobiotics are broken down to their inactivated metabolites. Conjugation is realized with some charged species such as sulfate, glycine, glutathione (GSH) or glucuronic acid. Sites on drugs where conjugation reactions occur include hydroxyl (-OH), carboxyl (-COOH), sulfhydryl (-SH), and amino (NH2) groups. Less active conjugation reaction products have increased molecular weight unlike phase I reactions. Reactive electrophiles are detoxified by the addition of GSH, large anionic groups. Moreover, they produce metabolites with high polarity, whose catalysis is a large group of broad-specificity transferases and through active transport and membranes. This includes nucleophilic or electrophilic groups which in combination can metabolize almost any hydrophobic compound that contains. The glutathione S-transferases (GSTs) are the most well-known class of this group [57].

2.3.3. Liver Disease

The liver is an organ placed below your rib cage on the right side of the abdomen, which plays an important role in digesting food and prevent the body from toxic substances. When it does not work efficiently, vital functions are affected negatively by the whole body. That

means that the liver exposes harmful effects or some bad factors which are either from environment or intrinsic. A variety of factors can cause liver disease. Infections sources by viruses, using alcohol, obesity, and genetic inheritance are common causes of liver diseases [58]. Moreover, cancers, immune system abnormality, fat accumulation in the liver (nonalcoholic fatty liver disease), some medications or over-dose using of drugs and herbs are other commonly seen causes of liver failures [59]. A definition of liver disease for American liver foundation "Liver disease is a life-threatening situation that needs emergency medical care" or the liver is an inability to fulfill their functions and responsibilities for the whole body. Liver failure has usually appeared for many years and increases their symptoms progressively. It is the end stage of many liver diseases. Nevertheless, a small number of acute liver disease occurs suddenly (shorter than 2 days) and it is hard to diagnose at the beginning. If liver insufficiency occurs, the liver can not work anymore and repair can not be useful to the liver. There are two types of liver disease for the duration of detection.

- Acute disease

The liver stops function within days or weeks. These patients have not been suffered from the liver of liver problem story of lives.

- Chronic disease

The liver has been exposed to more damaging factors which are maintained over time and it is a reason to stop fulfilling the functions [60].

2.3.3.1. Symptoms of liver disease

Liver disease is a severe case because the symptoms can not be seen rapidly. The most common and the previous symptoms are nausea, loss of appetite, fatigue, and diarrhea[61,60]. In addition, abdominal pain and swelling, yellowish appearance of skin and eyes (jaundice), itching, dark color in urine, pale stool, tendency to bruise easily are the other symptoms of the following steps of liver failure [62,63].

2.3.3.2. Causes of the liver disease

There are two types of liver illness. One of them is acute liver disease and the reasons are different from chronic liver failure. The symptoms appear suddenly and the liver functions are failed.

The acute liver failure causes are acetaminophen overdose. The viruses consisting of hepatitis A, B, and E, the Epstein-barr virus, cytomegalovirus, herpes simplex virus, reactions to some medications and herbal drugs, eating poisonous wild mushrooms, autoimmune hepatitis, Wilson's disease, acute fatty liver in pregnancy, septic shock, Budd Chiari syndrome, industrial toxins [64].

- Acetaminophen overdose

  Acetaminophen (APAP) is a widely known and used analgesic agent over the world. It shows its effect in a short time as it is absorbed fast from the intestine. Peak serum concentrations are indicated in 1-2 hours for tablets or capsules, for the liquid preparations, this item is less than the solid preparations. 20 percent of the ingested dose is exposed to the first-pass metabolism. The distribution process takes between 2 and 4 hours, depending on different dosage forms. Distribution volume is 0.9L/kg. Moreover, elimination happens by hepatic biotransformation in the liver. The elimination half-life is indicated at 1.5-3 hours, metabolized 90 percent doses are converted to inactive sulfate and glucuronide conjugates, secreted in the urine. Cytochrome P450 is the metabolism remainder and finalized highly reactive intermediary compound, N-acetyl-p-benzoquinone imine (NAPQI). In a healthy liver, the intracellular glutathione forms abound to NAPQI rapidly and are removed as mercapturic adducts within the urine. Paracetamol overdoses condition, more production of NAPQI might extinguish glutathione (GSH) stores. When the stores reach the critical grade NAPQI tends to bind to other protein molecules. Therefore, it causes damage to the hepatocyte and hepatic failure is appeared [65]. All reactions of acetaminophen pathway are shown in Figure 2.5.

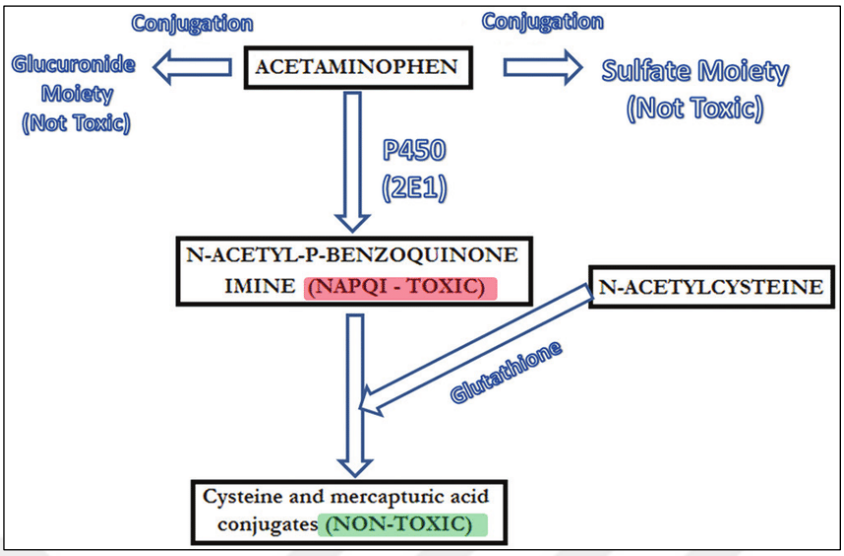

Figure 2.5. Acetominophen pathway [66]

APAP hepatoxicity appears after production of the noxious NAPQI metabolite in excess amount and causes the excess consumption of GSH, oxidative stress, and impairment of mitochondrial function. This leads to the extinguishing of adenosine triphosphate (ATP) stores. This means that APAP metabolic activation produces NAPQI which forms bound with more cellular proteins (especially mitochondrial). Binding depletes native antioxidant functions with the mitochondrial proteins which set the GSH depletion is crucial because increasing the mitochondrial ATP synthase α-subunit, causing ineffective ATP generations. Another mechanism of hepatotoxicity is the generation of toxic free radicals (peroxynitrite). GSH repletion ensures surplus cysteine for energy substrate in Krebs cycle and also helps of scavenging for free radicals and peroxynitrite. Mitochondrial suffer damage to their DNA by the toxic reactive oxygen and peroxynitrite molecules. They have acted in the process causing the cessation of ATP synthesis [67].

- Viruses

Viruses are the problem that causes an infection that inflames the liver. Some viruses types damage the liver Viral hepatitis is the most common cause. Hepatitis A has infected the body with unhygienic foods which are tainted by fecal. Eating and drinking something which has fecal residue, is a common way. Any symptoms can not be observed and it goes away by itself in 6 months, it can not damage the liver in a long term. Hepatitis B is infected by somebody else such as unprotected sex or taking drugs using the same needles. This condition carries on longer than 6 months, it causes liver cancer or other liver diseases.

Hepatitis C is infected by the blood Sharing needles and connection with virüs HIV are the common way of infection. Symptoms can not be observed for many years [68]. Chronic hepatitis C leads to cirrhosis and liver cancer. Hepatitis D is spread by contact with Hepatitis B patients and the transmission routes are the same ways. The last one is Hepatitis E is spread by contaminated water and nutrients. These are shown in Table 2.1 with names of causes and happens disease and effects.

Table 2.1. Causes of acute liver disease [69]

Causes of acute liver failure	
Infection	Hepatitis (A,B,C,D, nonA-nonB) CMV Epstein Barr virus Varicella
Drugs	Paracetamol Non-steroidal anti-inflammatory drugs Isoniazid Monooxidase inhibitors Ectasy
Metabolic	Wilson's disease
Circulatory causes	Congestive cardiac failure Cardiomyopathy Sepsis, shock Obstructive lesion of the aorta Venous occlusion (Pulmonary embolism)

The common causes of chronic liver failure are nonalcoholic fatty liver disease (NAFLD), infectious hepatitis, primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), alcoholic liver disease, and genetic conditions: hemochromatosis and alpha-1-antitrypsin deficiency (A1AD). These are shown in Table 2.2 with the name and their features and symptoms of the liver disease.

Table 2.2. Causes chronic liver diseases [70]

Etiology	Characteristics
Nonalcolic fatty liver dsease (NAFLD)	The most common form of CLD in the United States. Associated with obesity, metabolic syndrome, and insulin resistance Other potential causes include drugs(i.e., methotrexate, glucocorticoids), chemicals (benzene, xylene), growth hormone deficiency, hypothyroidism, nutritional deficiencies and genetic disorders
Infectious hepatitis	Can progress to NASH, a leading cause of cirrhosis in the United States Ccauses inflammation of the liver Over 400 million people worldwide are hepatitis B and 250 million people are affected by hepatitis C Viral causes of CLD in humans are hepatitis B, C and D, Epstein Barr virus (monoonucholesis), adenovirus, and cytomegalovirus
Primary biliary cirrhosis (PBC)	Affects up to I in every 4000 people and has a female-to-male ratio of 9:1 Autoimmune disease causing slow progressive destruction of small bile ducts leading to accumulation of bile (chelostatis) Tissue damage causes scarring and fibrosis May lead to cirrhosis
Primary scelerosing cholangitis (PSC)	Aotoimmune disease Inflmmation, scarring, and blockage of the bile ducts damages liver cells May lead to cirrhosis 10-15% lifetime risk of developing cholangiocarcinoma
Alcoholic liver disease	Consumption of large quantitis of alcohol over a prolonged period of time Ranges from asymptomatic to fulminant liver failure Cause of 8% of newly diagnosed cases of CLD and the combination of alcoholic liver disease and hepatitis C accounts for another 22% of cases
Genetic conditions: hemochromatitis and alpha-I-antithyripsin deficiency (AIAD)	Hereiteary disorder causing the body to absorb too much iron

	Symptoms typically develop at age 50-60 years Can cause damage to the liver, pancereas, and heart AIAD is an unusual cause of liver disease in children and adults

2.3.3. Liver Enzymes and Its Biological Changes in Acetominophen Overdose

More enzymes that regulate the vital functions in the liver are loaded into the liver. When liver failure is appeared the enzymes give some response. Evaluation of enzyme level in the liver and serum which is part of the blood is important to indicate liver damage [71]. However, elevations in enzymes are an important indicator of liver damage, these changes are also predictive of other important diseases or system disorders. Evaluation and indicate all of these some tests are performed, liver function test or liver enzymatic tests. These are alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, and bilirubin level tests [72].

2.3.4.1. Alanine aminotransferase (ALT)

Alanine aminotransferase (ALT) is responsible as catalyze to produce the hepatic metabolite oxaloacetate which is formed by the transformation of amino groups [73]. The ALT enzyme is found to a large extent in the cytosol and hepatocytes [74,75]. ALT is active in the liver more than 3000 times than in the serum. Therefore, hepatocellular damages or death cases, starting release ALT from the liver to serum. Thus, with increasing ALT levels, the activity of the enzyme in serum increases. However, it is especially found in the liver, ALT is placed in the kidney, heart, and skeletal muscle in small quantities [76]. In acute cases, AST level in serum increases suddenly, a higher level than ALT due to AST activity in the liver and release in hepatic injury. ALT has a longer plasma half-life so in 24 to 48 hours in damaging happens ALT will become higher than AST, for chronic cases, ALT is more increased than AST but in the fibrosis progresses, AST to ALT ratio is increased with the activity of ALT is decreased. As a result of this, cirrhosis appears, AST is much amount in the serum [77].
ALT level is an important enzyme blood test that is used to detect liver disease and also provides easy application due to low-cost and ready diagnose of liver failure. Additionally, ALT is a very important test to determine asymptomatic viral hepatitis and non-alcoholic

fatty liver disease that are inapparent types of liver disease. Some factors affected serum ALT activity, caused by hepatic necrosis. The gender factor associated with body mass index (BMI) and triglyceride levels alters the ALT level in serum, which is known to be at a higher level in men when comparing with those in women [78,79,80]. Among men, ALT levels deal in a positive correlation with total cholesterol and consumption of alcohol however age, smoking cigarettes and regular exercise have a negative correlation with it [81,82]. Glucose levels increase, whereas oral contraceptives decrease ALT activity in serum for women [76].

ALT is an indicator of liver illness. Rising serum ALT levels are also a sign of hepatocellular injury and identify an ongoing liver disease process. If there is ALT level elevation in serum with fatigue, anorexia, or pruritus, the possibility of liver disease can increase. There is more study about fatty liver disease, nonalcoholic liver disease, or viral hepatitis and associated with the diagnosis time and ALT level [76].

2.3.4.2. Aspartate aminotransferase (AST)

Aspartate aminotransferase (AST) is a biological catalyst like ALT in the liver [83]. The reversible transfer of an amino group between aspartate and alpha-keto acids is catalyzed by AST [84]. AST is primarily placed in the liver. Heart cells, muscle tissues, red blood cells, and other organs, such as the pancreas and kidneys also have AST. The elevation of AST is used for the diagnosis of liver illness. Additionally, the changes of the level are one parameter for the other tissue of liver damaged. The normal concentrations of AST are 5 to 40 U I-1 in blood. the pathways of AST in the case of disease are released into the bloodstream from the liver, the level raises in blood. these are shown that the tissue-damaging appears. After the severe disease, the AST level raises to standard level 10 times [83]. AST level raises to the highest level in other diseases such as skeletal muscle disorders, muscular dystrophies, and inflammatory [83,84]. AST has two isoenzymes in eukaryotic organisms. Cytosolic GOT1/cAST is produced by using heart cells and red blood cells. The second one is mitochondrial GOT2/mAST which is placed in the liver [85]. AST is also increased in burns and heart procedures [86]. It produces the transamination reaction such as ALT enzyme and the product of this transamination is oxaloacetate and glutamate [87].
AST reactions need the cofactor specific for AST and ALT enzymatic reactions, the cofactor is pyridoxal-5-phosphate (P5P) or also known as activated vitamin B6. The bond type is covalent but reversibly to lysine. Accepting the amino group from the amino acid reaction and transferring it to the carbonyl molecules are catalyzed with lysine [88]. If the enzyme

does not have the bounding cofactor, P5P is added to serum or purified extract may show a marked increase in AST activity. AST is placed in many fluids and organs of the human body except urine. On the other hand, unlike ALT, it is placed in the heart, kidneys, pancreas, and skeletal muscle [89]. As a result, AST is both a cytoplasmic and mitochondrial isoenzyme. However, the cytoplasmic isoenzyme predominant in the serum may increase the mitochondrial one and rarely be higher than the cytoplasmic one and is used in the diagnosis of the disease [90]. Although AST in serum levels increases in myocardial infarction it rises too slowly to be used for diagnosis [91].

2.3.4.3. Alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) is placed on the external layer of the membrane of a cell and it is a group of isoenzymes. They are catalysis of the organic phosphate esters in hydrolysis reaction with used zinc and magnesium as cofactors. However, ALP is located in different tissues and that have different physicochemical properties. ALP enzymes in the canicular membrane of hepatocytes are cytosolic and they are located in the placenta, ileal mucosa, kidney, bone, liver, and intestine in low concentrations but a high concentration of it is found in serum (80 percent) from releasing liver and bone [92,93]. ALP has tissue-specific enzymes and tissue nonspecific enzymes types. Tissue-specific enzymes are only placed in germinal tissue, intestine, and placenta, expression in physiological conditions but subscribe to ALP in circulation pool of the serum. The stimulation of serum production is increased. The tissue-nonspecific ALP, produced by the fraction circulating in serum, is of clinical interest. ALP in liver, bone, and kidney synthesis codes are responsible for a single gene. ALP which is located in the other tissue is encoded by different genes. However, this type of ALPhave different carbohydrates and lipid side chains, have the same amino acid chains. Therefore, post-translational modifications give different physicochemical functions to them [94,95].

2.3.4.4. Bilirubin

Bilirubin in bile is a brownish and yellow substance that is produced by destroying the aged red blood cells. The bilirubin is eliminated from the body in stool with giving the color to feces. In the body, two forms of bilirubin circulating in the blood. One of them is indirect or unconjugated bilirubin which is insoluble in water and moves to the liver in the bloodstream and is converted to soluble direct or conjugated bilirubin. The second one is direct or conjugated bilirubin, soluble in water, and is formed by the conversion of the indirect bilirubin in the liver. A rising bilirubin level is manifested by jaundice on the skin and the

white part of the eyes. Jaundice is caused by hepatitis, hemolytic anemia, or stopped bile ducts [96].

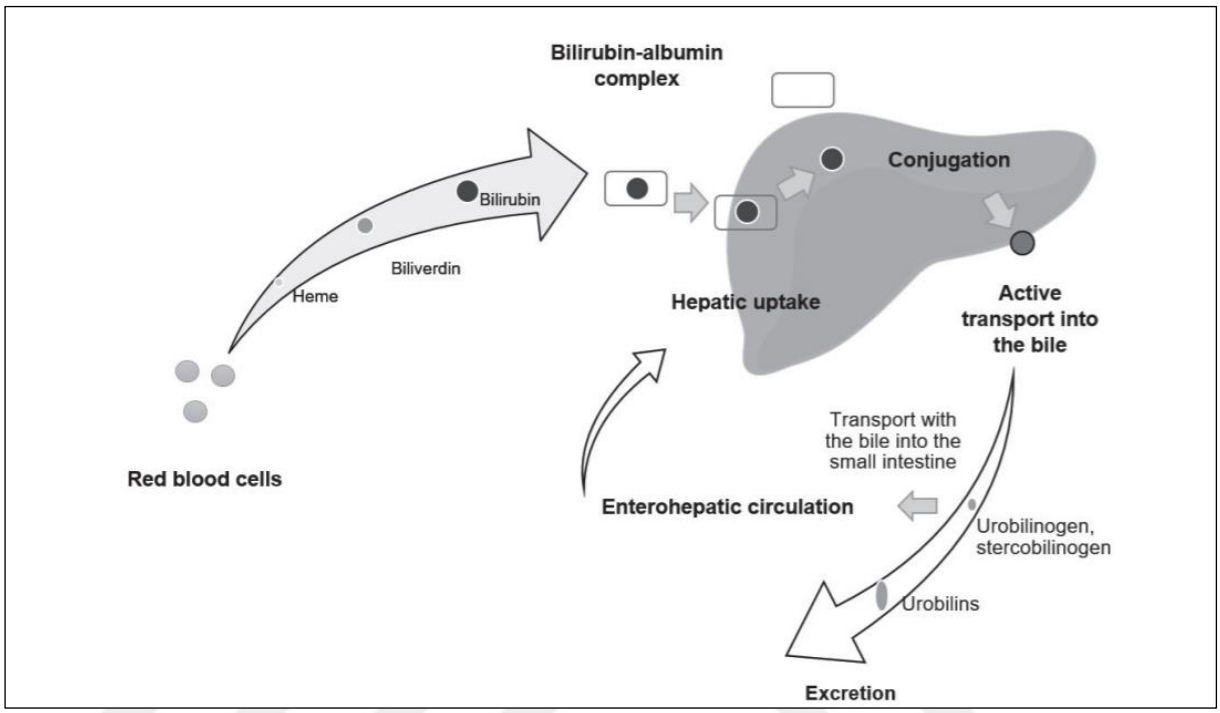

Figure 2.6. Bilirubin transport [97]

Bilirubin production metabolism is started with the degradation of heme by heme oxygenase in order to produce biliverdin, carbon monoxide, and ferrous iron [98]. Bilverdinis has applied the redox reaction and this relatively polar and nontoxic compound is reduced by using biliverdin reductase enzyme to bilirubin. This conversion process is reversible and conversion from bilirubin to biliverdin is performed by oxidation, resulting in amplification of the effects of biliverdin [99]. In addition, biliverdin is produced only by most of the vertebrates, bilirubin hypothesizes this but not biliverdin performed production. Bilirubin crosses the placental barrier due to hydrophobicity. As a result, this effect of bilirubin prevents biliverdin replacing in the fetus [100].

2.3.4.5. Albumin

Albumin is a crucial protein that is produced by the liver and transports nutrients and hormones to grow or repair the tissues. Additionally, albumin has high oncotic activity and prolonged half-life which gives an effective plasma expander in the intravascular region in the body. Therefore, it is used for the management of patients with cirrhosis and ascites

[101]. Colloid osmotic pressure is the major physiological function of albumin. Moreover, albumin has also crucial functions such as transportation of diversity molecules, ligand binding, antioxidant and anti-inflammatory actions [102]. It joins water cations, hormones bilirubin, fatty acids [103,10102]. When having surgery or burning the albumin level is low in serum, in addition to dysfunction kidney or liver is causes of the low albumin level [104]. Reduced albumin synthesis is one of the causes of cirrhosis but also posttranscriptional changes and oxidative damage which are a function of albumin and specific alteration tı its structure are also crucial causes of cirrhosis, impaired functions of the liver [105,106]. Albumin is an important role to observe in a range of established and propounded therapeutic indications in liver failure [107] and it is a primary plasma protein that standing for 50 percent of circulating proteins in a body [108]. Albumin synthesis is responsible for chromosome 4 and included 585 amino acids negatively charge on neutral pH [109]. Albumin has three structurally similar domains which have two subdomains each of them [110]. This structure provides stability and also allow to form of bond and transport molecules which are endogenous and exogenous [111]. Glycolisation, reversible and irreversible oxidation like enzymatic and non-enzymatic reactions can lead to modifications on the chemical structure of the albumin [112,113]. Albumin is produced by the hepatocytes and gives into the intravascular space. The ratio of the albumin is two-thirds is travel from hepatocytes to muscle and skin by blood and going into the circulation both capillaries and lymphatic system [114]. In healthy individuals, albumin is produced between 9 and 12 grams per day, and 20 to 30% of them are produced by hepatocytes [110]. Therefore, the liver has more functional storage which increases 3-4 times of required [115]. The circulatory half-life is 16-18 hours of albumin which is located from intravascular change with extravascular regions in the body. The overall half-life which is included catabolism and intravascular regions is prolonged at 9-12 days. However, in disease, the time is increased by the alteration of albumin [116].

2.3.4.6. ALT and AST ratios

The level of enzymes is a crucial sign of liver disease in addition to GGTP and LDH are another test to diagnose liver diseases. A GGTP tests gas to instances. One of them is ALP level is increased by conferring liver. The second one is AST/ALT ratio is increased (greater than 2). They are shown to have alcoholic liver disease [117]. These test combinations improve the single serum enzyme observations. An increasing AST and ALT in serum is an indicator of damaging organs such as the liver or kidney. Therefore, when the AST/ALT

ratio increases (bigger than 1.5), the consumption of alcohol is the reason for liver illness [118,119]. However, consumption higher amount of alcohol displays elevation aminotransferase levels that do not show a high ratio of AST/ALT. Some factors affected this ratio such as the seriousness of the liver illness [120]. The ratio is 2:1 which is shown setting the gamma-glutamyl transferase the other enzyme in the liver [121]. For alcoholic liver disease, the ratio reaches 2, for chronic hepatitis and chronic cholestatic syndromes are 1. The viral hepatitis is shown at the ratio, is liver enzymes [122].

2.3.5. Acetaminophen Overdose and NAC Therapy

Drug depended liver damage is commonly event coincide in clinical practice. A huge number of compounds are metabolized microsomes of the liver such as herbs and alternative medications. The most important cause of liver failure is the history of patients. However, liver failure is fulminant with hepatic encephalopathy and coagulopathy preceding jaundice [123]. In the World, acetaminophen (APAP), has been commonly used as an antipyretic or analgesic since 1955. Easily accessible in different dosage forms, low cost, and being over-the-counter medication are the most important factors in achieving this result [124]. APAP dose is an important parameter to the efficacy and health fort he humans life. For FDA reported as 4000 mg is safe for one day any toxic effect can not appear at this dose [125,126]. In some cases, APAP appeared toxic effect which is difficult to detect due to accessible several variety dose forms, combination supplements [125]. The first APAP hepatotoxicity was reported in the US. in the mid-1980s and all incidence was growing with the symptoms. The commonly used pharmaceutical drug was shown as a reason for the drug-induced liver injury [124,127,128]. More people are suffered from APAP toxicity all over the World. In the US 30000 patients are adopted to health centers or hospitals to take therapy and prevent hepatotoxicity [129]. Two of therapy are preferred liver transplantation ad NAC therapies. Liver transplantation is applied secondarily to APAP toxicity however NAC therapy is applied to the first APAP toxicity and mild incidence in the patients [125].

In NAC therapy, N-Acetylcysteine, a sulfhydryl donor such as cysteine, methionine, and cysteamine, is an effective antidote that is used to protect against APAP hepatoxicity [130,131,132]. Easy to apply and low toxicity is preferred to this treatment this incidence [133]. In addition, their known treatment mechanism and usage in the patient's study are

provided to preferred to treat the APAP overdose toxicity [134,135]. The hepatoxicity of APAP is protected by N-acetylcysteine in 4 ways. First one is that isolated hepatocytes are included in the synthesis of N-acetylcysteine which supports glutathione to the source of cysteine [136]. However, hepatocytes are incubated with APAP, very high concentrations of N-acetylcysteine, and the sulfhydryl are needed to enhance the production of acetaminophen glutathione [137]. The second mechanism of action is to adduct production between N-acetylcysteine and reactive APAP intermediate product, so taking an action against covalent binding and cell injury. Incubation process in microsome, the reactive APAP metabolite is ready to the product of sulfhydryl anions containing N acetylcysteine anion[138]. However, isolated hepatocytes which are incubated with APAP and N-acetylcysteine does not result in the production of the N-acetylcysteine to APAP [136,137]. Applying N acetylcysteine enhanced the production of the N-acetylcysteine of mercapturic acid overdose patients [139]. However, direct adduct formation is uncorroborated whether it is an important protective mechanism. As a third mechanism, increasing the accessibility of inorganic sulfate is protected by N-acetylcysteine. Therefore, in order to form toxic intermediate increasing the production of APAP-sulfate and reducing parts of the APAP are metabolized [140]. The last one is preventing the glutathione and covalent binding of the toxic intermediate product, covalent and decreasing reactive metabolite from cysteine or ascorbic acid [141].

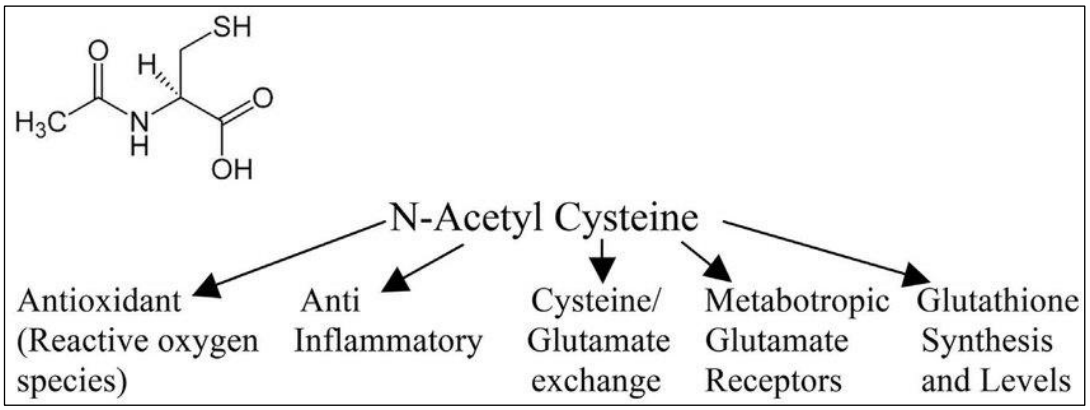

Figure 2.7. Mechanism of action for NAC [142]

2.3.6. AST and ALT Reactions

AST and ALT are the most important catalyst enzymes produced by the liver. They are called transaminase enzymes due to their reactions, transamination. Transamination reaction is performed by transfer the amino group (NH2) or (NH3+) from aminoacid types (α-aminoacid (2-amino acid) to α- ketoacid (2- keto or oxoacid) [143]. This reaction is determined by some methods with an indication of the reaction the photometric method is determined the ALT which is located in serum activity by using pyruvate and glutamate as substrates [144]. As a result of the transamination reaction between oxalacetate and l-glutamate, α-oxoglutarate is produced as a determinant of the AST level [145]. The AST activity is observed by the oxidation process of NADH and this is performed via using catalyzed malate dehydrogenase [146]. ALT and AST reaction is measured by linked of NADH/NAD+ systems. The two-color reaction is reduced co-enzyme NADH and colored formazan is formed by tetrazolium salt and transferring electron [147].

3. MATERIAL AND METHODS

3.1. PREPARATION OF NANOPARTICLES

Soy lecithin was mixed with water using a high-speed homogenizer for 5 minutes. This mixture was called a 2% soy lecithin mixture. The sonication technique by probe sonicator was then applied to this mixture 3 times at 15 minutes. These nanoparticles were lyophilized for one night to increase their stability [148].

3.2. PARTICLE SIZE AND ZETA POTENTIAL MEASUREMENTS

The obtained nanoparticles form of soy lecithin and their physicochemical features were characterized [149]. Average droplet size and PDI or polydispersity index value were measured by the method of Dynamic Light Scattering (DLS, Nano ZS, Malvern Instruments, U.K.) three times. The results were determined by averaging three sizes at 25°C by using disposable cuvettes. In addition, PDI was polydispersity index which shows homogeneity of the particles. The critical value of PDI was between 0.05 and 0.7[150,151]. The acceptable value of particle size was between 100-300 nm [152]. For determining the zeta potential disposable plain folded capillary zeta cells (Malvern Zetasizer Nano ZS) were used. The measurements were performed three times at 25±2°C to take average zeta potential. It means the charge of the particles and it is calculated by using the Helmholtz-smoluchowski equation in the process [150].

3.3. SILYMARIN DETERMINATION in HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The HPLC analysis was applied method with USP requirements. The samples were evaluated by HPLC (Waters07) system that included a UV spectrometric pump and detector. The column type was preferred Agilent ZORBAX Eclipse XDB-C18 column (4.6×150 mm,

5 µm). Two mobile phases were prepared according to USP. The mobile phase A contained methanol-phosphoric acid-water (20:0.5:80 v/v/v for 2 liters). The second mobile phase, called mobile phase B, contained methanol-phosphoric acid-water (80:0.5:20). The flow rate in this process was set to 1 mL/min and the injection volume was 10 µL [153,154].
Standard solutions were prepared using stock solution (1 mg / ml). Standard concentrations were 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.2 µg/mL, 15.6 µg/mL and 7.81 µg/mL. They were dissolved with methanol and the filtration procedure was performed with 0.45 µm filtrate [153].

In the first HPLC analysis step C18, the column was placed and the mobile phase switched to flow for column conditioning. Both reached 100% and the measurement was started by adjusting the mobile phase ratios during the analysis. The injection rate was entered as 20 µL from the vial to the column [153].

The calibration curve was prepared according to the measurement result used from the HPLC analysis. Measurements were of the area and concentration of the standard solutions [153].

3.4. PREPARATION OF *SILYBUM MARIANUM* EXTRACTS

The *Silybum marianum* seed contained silymarin. In the extraction process, three different *Silybum marianum* were used one of them was obtained local market the other one was standardized *Silybum marianum* seeds from firms and the last one was obtained pure silymarin from Sigma.
The extraction method was modified from the solvent extraction process and *Silybum marianum* seeds were weighed 5 g, added in 180 ml of petroleum ether. Then, seeds were boiled in the Soxhlet apparatus for 4 hours. During this process, oily substances were removed and filtered liquid parts. Then, 175 ml of absolute alcohol was added to the solid part. This solution was kept for 3 days in a dark area. Then put in Soxhlet apparatus and the extraction process was performed for 4 hours. This liquid part was separated from the solid part. This liquid part was evaporated in a rotatory evaporator. After the evaporation process, 175 ml of purified water was added to the balloon. Finally, the solution was freeze-dried [154].

This was called for the product by local market extract (LME) and another one which was produced by the same process and called the standardized seeds to extract (SSE). The standardized seeds were obtained by the firm capsule which was contained standardized 300 mg *Silybum marianum* seeds.

3.5. HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY EVALUATION

Silybum marianum two seed extracts were monitored by high performance thin layer chromatography (HPTLC) using a mixture of chloroform: acetone:formic acid (75:16.5:8.5) as mobile phase. The HPTLC glass plates were observed under ultraviolet light short wavelength (254 nm). Pure silymarin, seeds of local market silymarin extract, and standardized seed extracts solution were prepared in methanol. Extracts: methanol ratio was 2:1. The solution was loaded on glass plates and dried. After drying plates, HPTLC plates were dyed using NP, and to fix the spots accurately apply polyethylene glycol solution for the fraction spot on the layer. The procedure was repeated twice with two different application volumes of 10 µL and 20 µL to separate the spots clearly and make them more visible on the layer.

3.6. PREPARATION OF LIPOSOMAL *SILYBUM MARIANUM* EXTRACTS

The soy lecithin (2% w/v) dispersion was prepared using probe sonicator and freeze-dried (Christ) technique. *Silybum marianum* extracts were incorporated in SL dispersion. The *Silybum marianum* extract was freeze-dried and soy lecithin as a ratio 1:1 and added sterile water under homogenizer at 15000 rpm for 15 minutes by using Heidoplh (T25) and sonicated probe sonicator at 20-25°C 7% cycle 70% power. Finally, the nanoparticles were freeze-dried. It was stored at +4°C in the refrigerator. Subsequently, of the ten formulations prepared, the three best formulations were determined and their size dispersion profiles were analyzed. The three different extracts were taken 100 mg and they were added 100 mg of soy lecithin nanoparticles. They were mixed with a sonicator at 15 minutes with 10 mL sterile water. After the formulation was a freeze-dried process overnight [151]. The first

nanoparticles formulation which contained LME was called SL:LME. The second one contained SSE which was called SL:SSE. The third one was contained pure silymarin and called SL:PSIL.

3.7. DISSOLUTION TEST

A dissolution apparatus (Pharmatest) was used in order to determine the release profile of the three different best formulations, the paddle method (USP II) was also applied and in the process of the method, it was stated that the second apparatus should be used at rpm in 900 ml media and the temperature should be kept constant at 37 ± 0.5 degree celsius[155,156]. Moreover, 2% sodium lauryl sulfate was added and pH was adjusted to 7.3 by adding 1 N sodium hydroxide solution. For this solution of 4.02 g NaOH was dissolved in 100 mL water [157]. These all requirements were completed the process was started and continued for 6 hours. 1 mL of samples were taken at specific minutes for each three different formulations. The samples were evaluated in the HPLC method in USP. For standard 2.22 g of pure silymarin was dissolved in methanol. The silymarin release profile was plotted and evaluated by the HPLC apparatus [158].

The loading efficiency was calculated for the best optimized nanosilymarin formulation by using the following equations.

$$\%\text{loading efficiency} = \frac{\text{amount of measured of test drug}}{\text{amount of standard drug}} \text{x}100 \qquad (3.1)$$

3.8. LIVER ENZYMES ASSESSMENT

In this study, a standard liver enzyme assessment process was performed for the collection of serum. ALT and AST levels were measured by using of Idexx Laboratory Automatic Biochemical Analyser [157]. The serum samples were put in the analyzer with the help of a pipette tip (Vet test) and ALT and AST test kits (Idexx Vet Test) were used.

3.9. *IN VIVO* STUDIES

Rats were randomized to four groups (n=6 rats/group) [158]. While assigning animal groups, young, male, similar weight, and Sprague Dawley type rats were preferred. Firstly, basal blood was taken from rats in all study groups and evaluated biochemically [159]. Rats in the first, second and fourth groups were orally administered a high amount of acetaminophen suspension (1500 mg/kg) for two days to cause liver injury [160,161]. After that, the blood samples were taken and evaluated with biochemical tests. When liver damage is determined, one group of rats was orally given with an aqueous dispersion of silymarin nanoparticles. The second one was applied with pure silymarin (prepared by adding silymarin to sterile water). Their content was triturated and prepared the suspension with sterile water. The third group was the control and the rats fed adlibitum application. The last one was not treated with any substances. The blood samples were collected from rats by jugular vein under anesthetic conditions. Blood samples were kept at 1 hour at room temperature. Then, serum was separated and placed in a heparinized tube (BD Vacutainer, SST II) from the other blood contents by applying at 4000 rpm for 15 minutes and at 4°C centrifugation [Hettich, Zentrifugen, Universal 320 R) [162]. Hepatoprotective activity was evaluated by serum ALT and AST levels [163]. Rats were sacrificed on day 6 of the studies. The blood samples were taken and the sacrification process was done under the anesthesia condition by using isoflurane. The liver samples were stored in 10% formalin solutions [164]. The histopathological studies were performed to determine the pathological changes in the different groups and liver by classical pathological methods [165]. The largest right lobe of the liver of the rats was excised and it was performed for the pathological and liver test studies [166].

3.10. HISTOPATHOLOGICAL STUDIES

Rats completing the experimental period were sacrificed under high-dose anesthesia. Liver tissue samples were put in 10% formol solution at +4°C. After the fixation procedure, tissues were cut into small pieces and wash in tap water for 1 night and dehydrated with increased alcohol series (70-80-90-96-100%). Then, it was made transparent in xylene and was

embedded in paraffin, and blocked. The block was trimmed and taken sections at thickness was 5µm using Leica RM 2245 microtome. In order to detect the lesions in the sections, sections were taken in the depth of the blocks. They were stained by hematoksilen-eosin and trikrom and all sections were observed in Leica light microscope. When the histopathological evaluations of organs were performed in the light microscope, each organ was evaluated for different parameters and scores in itself for the liver.

3.11. STATISTICAL ANALYSIS

Calculations of mean values and average standard deviations (SD) were performed. Analysis of data was performed by GraphPad Prism v. 5.04 program (GraphPad Software Inc., La Jolla, CA). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey analysis. $p<0.05$ was considered as statistically significant.

4. RESULTS AND DISCUSSIONS

4.1. CHARACTERIZATION OF SOY LECITHIN NANOPARTICLES

The characterization of nanoparticles is the crucial for oral route. Characterization values included size distribution, the zeta-potential, charge of the particles and PDI value shows the homogeneity of particles. Their physicochemical properties affected the disintegration, dissolution, and release profiles, and also stability and activity of materials on the cell and process in the body [167]. The nanoparticle size must be 100-300 nm for oral applications. The size distribution was shown in Figure 4.1. The measurements were performed three times for each cycle.

Nanoparticles were prepared by probe sonication technique at 25°C in different minutes. Physicochemical parameters cannot be ignored in the development of new nanocarrier systems. This method has been widely used for the nanoparticle [168]. In this part of the study, different minutes of sonication were tried to achieve the best formulation and as a result, their size, zeta potential, PDI value, and conductivity were measured and compared, and the preferred minutes was 15 (Table 4.1). The smallest size of nanoparticles was obtained in the 15 minutes by using a probe sonicator (101.7nm) with 0.278 PDI which displays the stability of the samples with colloidal suspension or aqueous solutions [169]. Additionally, it was measured 66.6 mV negative charge, 0.155 conductivity properties. In a study, using the cross-linker polymers to decrease particle size and PDI value were reported [170,171]. The sonication does not need any chemicals to provide these properties for preparing the nanoparticles. Therefore, this was preferred for the oral silymarin nanoparticle as a drug in order to obtain effective nanoparticles with absent increased toxicity chemicals.

Table 4.1. The size, zeta potential, conductivity, and PDI value for different minutes

Nanoparticle	Average size (nm)	Zeta potential (mV)	Conductivity (mS/cm)	PDI
Soy lecithin (minutes 0)	383.5	-55	0.0826	0.635
Soy lecithin (minutes 5)	144.9	-25.7	0.197	0.671
Soy lecithin (minutes 10)	107	-56	0.163	0.235
Soy lecithin (minutes 15)	101.7	-66.6	0.155	0.278

The values were acceptable for oral applications, so the technique, decreasing the size was effective on the nanoparticles and provides homogeneity and negatively charge to go into the cell easily [172,173]. This size distribution graph of the nanoparticles was shown (Figure 4.1).

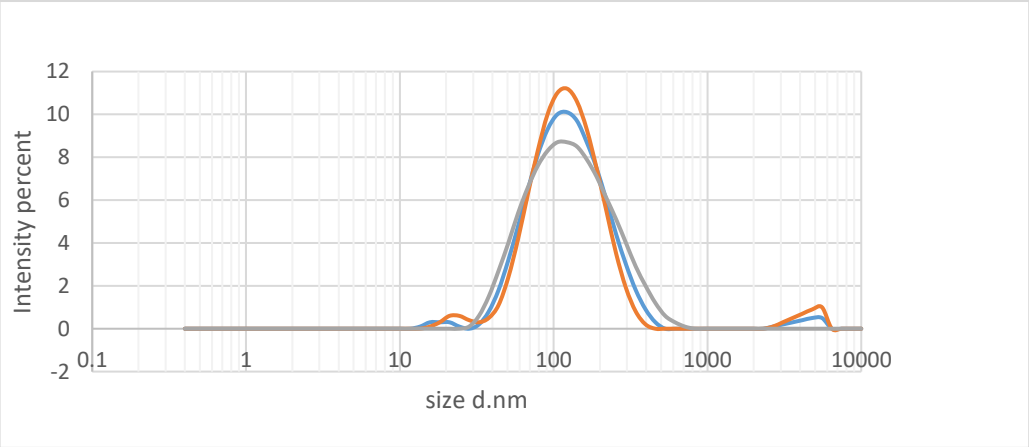

Figure 4.1. Size distribution of the nanoparticle three times for 15 minutes

After determining the 15 minutes the *Silybum marianum* extract was loaded to this nanoparticle. The soy lecithin nanoparticles were freeze-dried. The ten formulations were performed with the different amounts of soy lecithin and *Silybum marianum* extracts, the ratio of 1:1 was preferred as the best formulation. The extracts were obtained from local market, marketed products (standardized seeds), and pure silymarin from firms materials. The extracts were used and the measurements were performed. The results of silymarin nanoparticles were shown in Table 4.2.

Table 4.2. Size, zeta potential, conductivity, and PDI of silymarin nanoparticle

Nanoparticle	Size (nm)	Zeta potential (mV)	Conductivity (mS/cm)	PDI
SL:LME	1155	-52.9	0.0317	0.861
SL:SSE	138.9	0.0340	0.0209	0.383
SL:PSIL	379.2	-35.8	0.0185	0.450

SL:LME is nanoparticle with local market *Silybum marianum* seed extracts, SL:SSE is nanoparticle with standardized *Silybum marianum* seed extracts, SL:PSIL is nanoparticle with pure silymarin.

The measurements were performed three times and these results were average values. The best formulation was preferred SL:SSE which was contained the standardized seeds.

4.2. HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY ANALYSIS

Silymarin extracts were used as reference material for qualitative identification by HPTLC. The identity of the silymarin in the test solutions of the extract which was obtained from *Silybum marianum* seed was evaluated by comparing the RF with the standard alone silymarin formulation. After the two blue zone colors derivatives of the standards and the sample test solutions (silybin A and B) corresponding to were compared in HPTLC plates. HPTLC analysis proved the presence of silybin A and B, the major components of silymarin, in extract samples obtained from the seeds of two different companies.

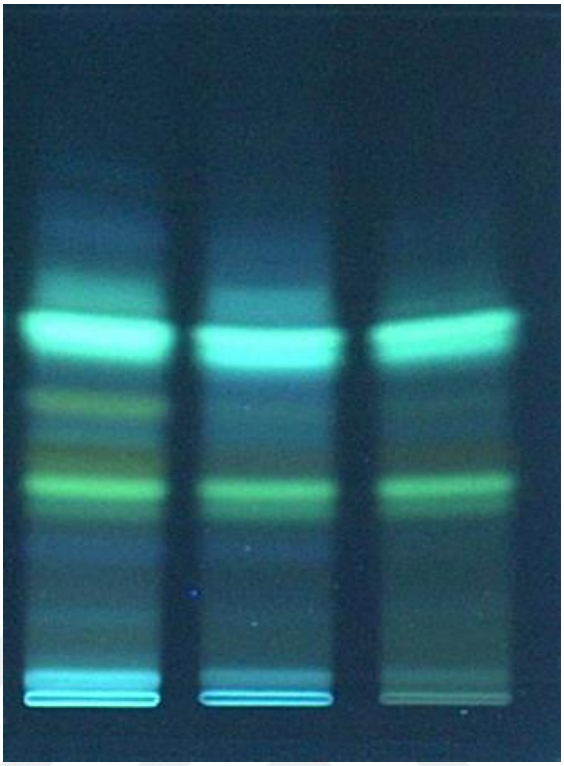

Figure 4.2. HPTLC results. The first column was shown of the reference or standard sample, alone silymarin, the second one was shown the standardized *Silybum marianum* seed extract and the extract of local market seed for the last one.

In some HPTLC studies of silymarin, the zones' colour and retention time were similar to our results [174,175]. The pure silymarin and our extracts gave the same zone for the more materials in them silybin [176]. This part of the study aims to prove the similar content between pure silymarin and prepared extract obtained from the standardized seed and local market. Silymarin was determined under UV-365 nm by two intense green-blue fluorescent zones of silybin/isosilybin silychristin [177]. These are similar to our findings.

4.3. CALIBRATION CURVE

The calibration curve was fitted by using pure silymarin. The different concentrations were prepared by using methanol the mobile phase was prepared after the analysis was performed in HPLC. As a result, the calibration curve was fitted (Figure 4.3.).

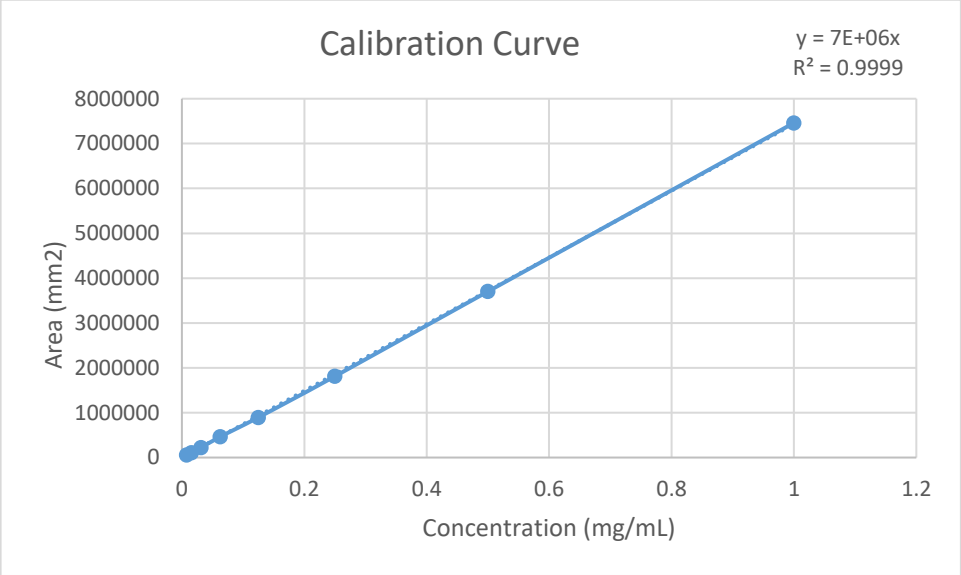

Figure 4.3. Calibration curve

Eight standard pure silymarin solutions with different concentrations were used to prepare the calibration curve. The solutions contained pure silymarin: 65-80% of Silybin A and B, Isoslybin A and B, Silydianin, and Silychristin. Isosilybin A and B, silychristin, silydianin, and other materials were not shown in the calibration curve due to their negligible level of absorption. Moreover, in the method of USP only silybin A and silybin B were evaluated. Preferred concentration absorption results were in the range of calibration curve for the high amounts of substances. The other substances' results may be far away from the range and they were examined and do not use to generate the calibration curve. The calibration curve was constructed with the changing concentration and their area from evaluated from the absorption [178]. The analysis showed the determination of the 95 percent confidence interval for the cut-off values and correlation coefficient (R^2) for the goodness of fit. The results were evaluated and using the Statistics Designer function within the software program [178,179]. The fact that R^2 is so close to 1 indicates that the results and measurements of the analysis performed are very accurate. In addition, indicating the acceptable linearity of our method [180]. The equation was used for the release profile in the dissolution test the measure the release amount in the media, and also these methods were validated for the dissolution test. A representative equation for the calibration curves is $y=7*10^{-6}x$ where y indicates the ratio of silybin A and silybin B peak area and x indicates the ratio of known concentration of silymarin [180]. A new sensitive method of determining

the silymarin in HPLC to carry out to analyze capsules with no interference from common excipients [181]. In a recent study, the extraction rate of silymarin was increased with boiling water and ensured that all components were precisely determined [182]. The separation efficiency was best so all materials were determined in separate peaks and detected accurately for USP method [183].

4.4. DISSOLUTION TEST

Crucial effects of solubility on absorption and bioavailability of drugs. Silymarin solubility is very low in plain water at 25°C, was found to be 50 µg/mL indicating [184]. The dissolution process was applied to the three best formulations. The dissolution process was performed with the USP method. The dissolution of the drug from the nanoparticles preparations was compared with each other. They were prepared by using LME, SSE, and PSIL. The pH of the media in the analysis that lasted for 6 hours was 7.3. The samples were taken and analyzed in HPLC validated method. The result was shown as the release rate of the oral formulation, in Figure 4.4 in this process.

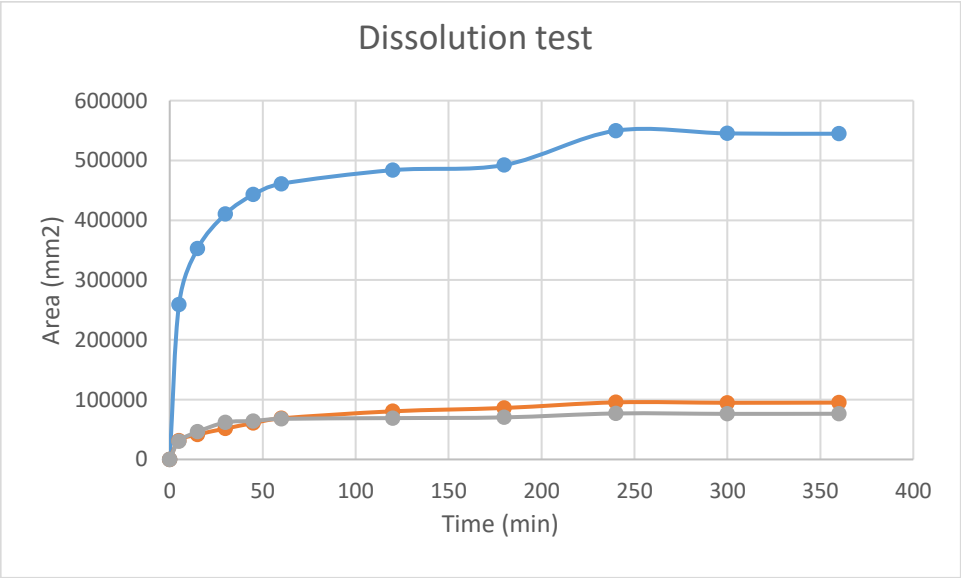

Figure 4.4. The dissolution rate of three formulations blue line was shown nanoparticle with pure silymarin (SL:PSIL), the orange line represented nanoparticle with standardized *Silybum marianum* seed extracts (SL:SSE), and the grey one was shown nanoparticle with local *Silybum mariaum* seed extracts (SL:LME).

The release rate showed that these preparation releases in a controlled manner. This was very important because the silymarin solubility and absorption were very low in the body so in a controlled release the silymarin dissolves in a low amount and absorbs easily and the low dose was so effective [185]. In addition, adverse effects and toxicity can decrease for the body.

The SL:SSE and SL:LME were significantly different $p<0.05$ than SL:PSIL formulation. The release rate is controlled manner. Therefore, both of them are coated the soy lecithin homogenously. As the hydrophilic parts, the loaded drug presents dissolve rapidly [186].

Pure silymarin can not go into the nanoparticle completely. In particular, about 80% of the drug was rapidly released from the nanoparticle within 15 minutes. Moreover, the dissolved drug concentration in the nanoparticles within 1 hour was about two and three-folds higher than when compared with SL:PSIL which was not encapsulated. Results show that drug release from the nanoparticles was achieved as slowly, controlled released, and completely. The nanoparticles have ultralow interfacial tensions and a large o/w interfacial area, so weak water-soluble drugs like silymarin were easily incorporated into the nanoparticles [187]. Thus, nanoparticles have an advantage in maintaining solubility, which ensures higher absorption in comparison with the normal formulations which is not encapsulated with nanoparticle formulations [188]. In another study, at pH 7.4 which was stimulated the intestinal area, between 8 and 13 percent drug was released in 1 hour, and also this release rate was observed as the controlled release. Similarly, in this study, both of the formulations were released in a controlled manner. The release drug amount in 1 hour was close to another result which was found from Bhavin et al. study [189]. The dissolution profiles of SL:LME, SL:SSE and SL:PSIL were compared using the evaluating the released amount of drug [190].

The loading efficiency was calculated 90.47 percent for SL:SSE which was used with the standardized *Silybum marianum* seed extracts. The other formulation studies were given in Table 4.3.

Table 4.3. Loading efficiency of the formulations

Formulations	% results		
	Silybin A and B	Silybin A	Silybin B
SIL	42	16	26
SL:PSIL	34	12	22
SL:SSE	38	14	24
SL:LME	26	9	17

SIL is silymarin, SL:PSIL represents nanoparticle with pure silymarin, SL:SSE represents nanoparticle with standardized *Silybum marianum* seed extracts, SL:LME represents nanoparticle with local *Silybum marianum* seed extracts.

4.5. *IN VIVO* STUDIES

Silybum marianum, or its active ingredient silymarin, has been used from ancient times in Chinese and Western communities to heal liver disorders and protect the liver from toxic substances. ALT and AST levels on the liver often play a decisive role in vital biochemical markers in liver damage. Increased amounts of AST and ALT in serum are signs of dysfunction and loss of integrity of the membrane. Acetaminophen-induced liver damage is further assessed by the release of these enzymes into the systemic circulation through the liver cell membrane [191]. An increase in levels above the normal limit indicated liver damage [192,193]. The silymarin level affected the plasmatic ALT and AST in the liver cells of ill rats. The level of these enzymes was investigated when they were healthy after being induced by acetaminophen and the last one was evaluated after treated silymarin formulations of ill rats. The effects of silymarin on plasmatic ALT and AST are represented in Figures 4.5 and 4.6 respectively. These evaluations showed that a statistically significant between all control and treatment groups. However, significant increases (40 percent resp.) in ALT levels after induced by acetaminophen, in both treatment groups, was verified, whereas silymarin treatment was approached to normal levels (Figure 4.5).

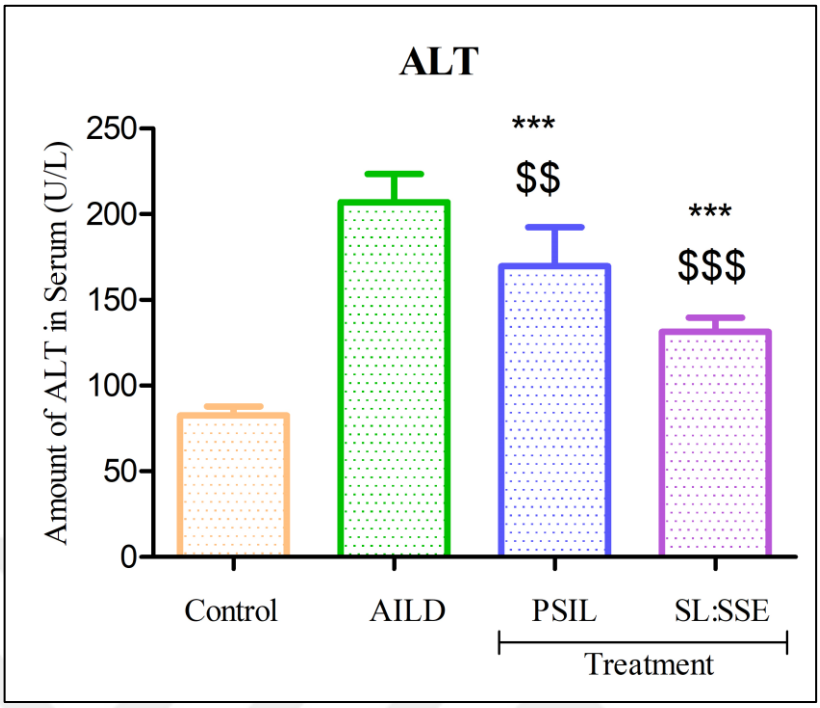

Figure 4.5. Changes of ALT level in rats serum

AILD: Acetominophen induced liver disease, PSIL: Pure silymarin, SL:SSE: Nanoparticle with standardized *Silybum marianum* seed extracts.

* and $ represents significantly lower than the APAP-treated animals at this time point

* significant difference compared to control group,*** p<0.0001,

$ significantly different compared to an ill group, $$$<0.0001, $$<0.001

In one study, all formulations containing silymarin and nanosilymarin investigated the treatment effect on plasmatic ALT level after induction as seen in Figure 4.5. The silymarin level was reduced from about 200 U/L to 150 U/L. In our study, it proved that the decrease was a statistically significant difference, and the levels decreased significantly like Apel et al. study. For this reason, nanosilymarin formulation has higher bioavailability than alone silymarin formulation.

In another observation, the effects of nanosilymarin formulation on serum levels of biomarkers compared to silymarin solution without nanoparticles were investigated. The results of the studies show that the nanoparticles of the silymarin formulation lower the enzyme level more than the silymarin formulation. It has been reported that the formulation of nanosilymarin is more convenient than the controlled release profiles, and therefore, is

more effective than other suspension or capsule forms and increases bioavailability and solubility in the body.

Furthermore, silymarin is been known to be essential in the tendency to lower serum ALT levels in liver damage [194]. These results reported that the nanoparticle silymarin formulation is compatible with our values, and this proves our results. Therefore, the bioavailability and solubility of nanoparticle formulations of drugs that could not be absorbed orally, and study of Yen et al. has been conducted to investigate and show similar findings on this manuscript [195].

Another vital liver enzyme is AST. High AST level liver in addition to liver damage, may be an indication that it has diseases such as viral hepatitis, cirrhosis, anemia, cardiac infarction, or skeletal and muscle injury [196]. AST value can vary between 8 U/L and 50 U/L depending on the gender difference in healthy individuals [197]. In this study, the varying levels of AST that suffered damage from acetaminophen and treated with two different formulations of silymarin are been shown in Figure 4.6.

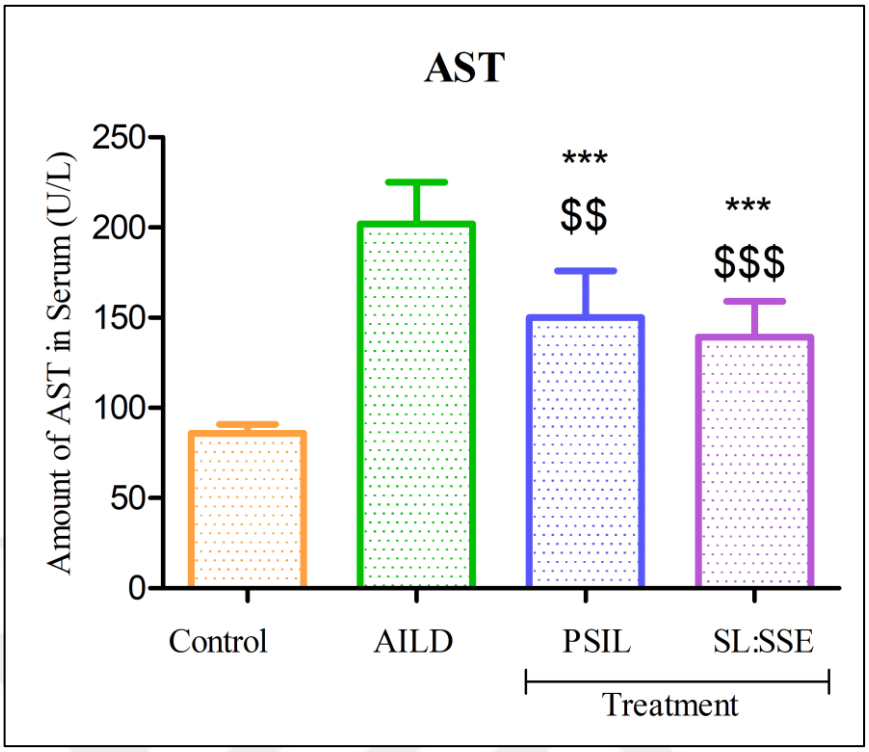

Figure 4.6. Changes of AST level in rats serum

AILD: Acetominophen induced liver disease, PSIL: Pure silymarin, SL:SSE: Nanoparticle with standardized *Silybum marianum* seed extracts.

* and $ represents significantly lower than the APAP-treated animals at this point

* significantly different compared to control group,***p<0.0001

$ significantly different compared to ill group, $$$ p<0.0001, $$ p<0.001

Liver damage, cell necrosis, and a decrease in GSH detoxification capacity may occur in the following states due to high dose acetaminophen Liver damage, cell necrosis and a decrease in GSH detoxification capacity may occur in the following states due to high dose acetaminophen. The results of this study also decreased the serum level of silymarin with statistically significant differences. In addition, in our study, the nanoparticle form of silymarin decreased the level more than the suspension silymarin compared to the control. When the formulation of nanosilymarin is examined appropriately, it has significant differences ($p<0.01$) from alone silymarin ($p <0.05$) [198]. Moreover, Wang et al. studied the effects of silymarin and nanoforms of silymarin on ALT and AST levels in the serum. As a result, the nanoform of silymarin decreased the level of ALT and AST more than the pure silymarin and their tablets. These were reduced hepatotoxicity and evidence the

nanoform of the silymarin more effective to reduce the biochemical marker in the serum and side effects that can cause toxicity [199]. One study reported that the bioavailability of nanoparticles of silymarin was higher than the suspension form of silymarin [200].

The results of this study also decreased the serum level of silymarin with statistically significant differences. In addition, in our study, the nanoparticle form of silymarin decreased the level more than the suspension silymarin compared to the control. When the formulation of nanosilymarin is examined appropriately, it has significant differences ($p < 0.01$) from pure silymarin ($p < 0.05$). ın the current study, the nanoforms of silymarin showed that in a controlled release and the bioavailability was high [201].

Abdulrazzag et al. studied the effect of ascorbic acid, silymarin, and combination induced by APAP in the liver. Although it was observed that the combination formulation with ascorbic acid and silymarin significantly reduced the AST level in the serum, it was determined that the silymarin formulation alone decreased the AST levels with a significant difference [202].

Moreover, Mansour et al. studied the healing effect of silymarin on cisplatin-induced liver disease. Cisplatin is an anticancer agent. The study showed that silymarin has protective effects from acetaminophen-induced (damaged liver) and treats liver injury after drug administration [203]. In our study, it was observed that after the drug was administered, the formulation of nanosilymarin significantly decreased the AST level in serum.

4.6. HISTOPATHOLOGICAL STUDIES

Histopathological studies prove the results of the analysis of biochemical parameters. Histological analyzes of rat liver treated with silymarin after APAP was induced showed a significant reduction in hepatotoxicity. The livers were analyzed according to interstitial edema, occlusion in the synozoid capillaries, hepatocellular necrosis, vacuolar degeneration, and leukocyte infiltration. Following APAP-induced hepatotoxicity, in all groups, intense infiltration of inflammatory cells was shown around the central vein and cellular borders were damaged [204]. When the liver tissues were compared, it was observed that the group treated with nanoparticles of silymarin was healthier than the group treated with pure silymarin and that tissue damage was more repaired. The tissues were shown in Figure 4.7.

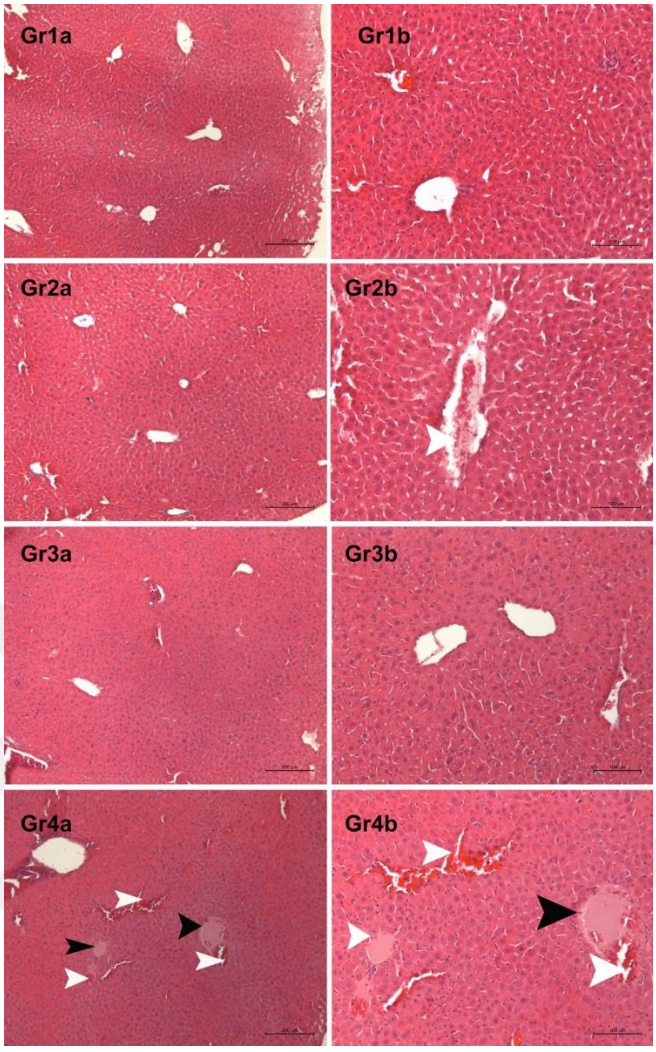

Figure 4.7. Histopathology of livers 24 hours after acetaminophen (APAP) injection. The livers were collected 24 hours from all groups after APAP administration (1500 g/kg). Panels (a): the tissues were observed at 200 µm and panel (b): the tissues were observed at 10 µm. The first group received APAP and treat nanosilymarin formulation respectively, the second group treated APAP and treat pure silymarin third group was the control they fed up with adlibitum and the last or fourth group received only APAP. (APAP, 1500 g/kg per 2 two hays, SLM, 100 mg/kg per 3 days).

Nanosilymarin formulation showed the controlled release and enhanced the bioavailability of silymarin. Therefore, nanosilymarin of the formulation was more effective than the suspension form of silymarin formulation in one study [205]. Furthermore, one study reported that the nanoparticles silymarin low dose more effective than the pure silymarin high dose, and the histopathological results were evidence of nanoparticles effectiveness.

These results supported to current study [206]. The study of Singhal et al. on the biochemical parameters was supported to prove histopathological results. The rats were induced by APAP. When the hepatotoxicity that occurs was characterized, interstitial edema, congestion on the sinusoid capillary, hepatocellular necrosis, vacuolization on hepatocytes, and hepatic tissues inflammation including the presence of moderate infiltration of neutrophils [204]. In histopathological part of observations was consistent with our images. These observations were supported each other as the histopathological analyses.

The one study on liver cancer and effects of nanoforms of silymarin on the liver cells. In the study, it was observed that silymarin corrected completely damaged liver cells and at the same time, bleeding was observed in liver cells treated with pure silymarin, while there was no bleeding in the nanoparticle applied liver cells. As a result of this study, the nanoforms of silybin were more effective than the normal silymarin [207]. These results were proved to our findings with the level of the biomarkers in the serum.

In some studies, nanoparticles of silymarin were more effective in histopathological analyses. The nanosilymarin and pure silymarin were applied to liver damage rats. The nanoparticle of silymarin was effective than pure silymarin. While it was observed that there was damage in the liver of the heat to which pure silymarin was applied, no damage was observed in the liver when nanoparticle silymarin was applied. According to another study, results showed that nanosilymarin treatment had been more effective on the liver injury when compared to other groups. These results were proved our result in the study [208].

5. CONCLUSION

Recently, more part of the population is suffering from liver disease. They are looking for a more effective, natural, and new treatment strategy. For this reason, studies are carried out to maximize the usefulness of the natural herbs, which have been ongoing since ancient times, by combining them with today's new technologies, minimizing the side effects and dosage. The *Silybum marianum* is one of the important plants of this. It has been used for a long time and is known effective in hepatic disease. In this study, novel technology as nanotechnology and silymarin which are the actives of *Silybum marianum* extracts were combined. The standardized seeds and soy lecithin were used and prepared the nanoparticle with the recent methods. The tests were performed and the best formulation was determined and the *in vivo* and histopathological studies have been continued with the best formulation. The data gathered from this study indicated that the nanoparticle formulation of silymarin was more effective than pure silymarin formulation. Furthermore, *Silybum marianum* extract was encapsulated by soy lecithin nanoparticles in order to treat induced liver damage with a low dose. Overall data suggested that the nanoparticles provide the controlled manner release, low dose, and more effectiveness. This technology was used for the lower soluble drugs in water as silymarin. However, more studies are required to observe the surface of the nanoparticles and determine how long it takes to release all of the encapsulated amounts. To increase the effect of silymarin different nanoparticle techniques will be performed.

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YEDİTEPE ÜNİVERSİTESİ

T.C. YEDİTEPE ÜNİVERSİTESİ, DENEY HAYVANLARI ETİK KURULU (YÜDHEK)

ETİK KURUL KARARI

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
13.02.2019	725	11.01.2019	Dr. Öğr. Üyesi Gülengül DUMAN

'Silymarin içeren nanopartikül formülasyonu geliştirme ve hepatoprotektif etkisinin değerlendirmesi' adlı bilimsel çalışma etik kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oy birliğiyle karar verilmiştir.

Etik Onay Geçerlilik Süresi: 3 Yıl	**Hayvan Türü ve cinsiyeti:** Sıçan ♂	**Hayvan Sayısı:** 24

GÖREVİ	ADI SOYADI	
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Yardımcısı	Prof. Dr. Erdem YEŞİLADA	
Raportör	Vet. Hekim Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Prof. Dr. Rukset ATTAR	**KATILMADI**
Üye	Doç. Dr. Soner DOĞAN	**KATILMADI**
Üye	Doç. Dr. Ediz DENİZ	
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