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INSTITUTE OF HEALTH SCIENCES PHARMACEUTICAL
TOXICOLOGY DOCTORAL PROGRAM

***EXAMINATION OF EPIDERMAL GROWTH
FACTOR TREATMENT IN AMATOXIN INDUCED
HEPATOTOXICITY***

DOCTOR OF PHILOSOPHY THESIS

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DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

13/06/2023

Sinan KARACABEY



BEYAN

Bu tezin kendi çalışmam olduğunu, planlanmasından yazımına kadar hiçbir aşamasında etik dışı davranışımın olmadığını, tezdeki bütün bilgileri akademik ve etik kurallar içinde elde ettiğimi, tez çalışmasıyla elde edilmeyen bütün bilgi ve yorumlara kaynak gösterdiğimi ve bu kaynakları kaynaklar listesine aldığımı, tez çalışması ve yazımı sırasında patent ve telif haklarını ihlal edici bir davranışımın olmadığını beyan ederim.

13/06/2023

Sinan KARACABEY

DEDICATION

I would like to dedicate this thesis to...

My beloved Family for their endless love, sacrifices, supports and advices.

**My mentor, Hande Sipahi, I could not have done this without you. Thank you for
all of your support.**

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LIST OF SYMBOLS AND ABBREVIATONS

α -AMA: Alpha Amanitin

ACTH: Adrenocorticotrophic Hormone

ALP: Alchaline Phospatase

ALT: Alanin Aminotransferase

AST: Aspartat aminotransferase

BHT: Butylhydroxytoluene

CCl₄: Carbon Tetra Chloride

CZE: Capillary Electrophoresis

DNA: Deoksiribo Nucleic Acid

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

ELISA: Enzyme-Labeled Immunosorbent Assay

ErbB: Avian Erythroblastosis Oncogene B

GABA: Gamma-Aminobutyric Acid

GGT: Gama Glutamyl Transferase

GH: Growth Hormone

HE: Hematoxylen-Eosine

HSC: Hepatic Stellate Cells

HGF: Hepatocyte Growth Factor

LC: Ultraviolet Combined Liquid Chromatography

LC-MS/MS: Liquid Mass Spectrophotometry

LD50: Lethal Dose 50

LHDS: Liver Histological Damage Score

MAPK: Mitogen Activated Protein Kinases

MDA: Malondialdehyde

mRNA: Messenger Ribo Nucleic Acid

MTT: 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2H-Tetrazolium Bromide

NAC: N-Acetylcysteine

NADPH: Nicotinamide Adenine Dinucleotide Phosphate Hydrogen

NGF: Nerve Growth Factor

OATP: Organic Anion Transporter Peptide

PBS: Phosphate-Buffered Saline

PI3K/Akt: Phosphatidylinositol 3-Kinase/Protein Kinase B

PRL: Prolactin

RIA: Radioimmunoassay (RIA)

ROS: Reactive Oxygen Species

SOD: Super Oxide Dismutase

SIL: Silibinin

TBA: Thiobarbituric Acid

TNF- α : Tumor Necrosis Factor Alpha

ABSTRACT

Sinan K. (2023). Examination of Epidermal Growth Factor Treatment in Amatoxin Induced Hepatotoxicity. Yeditepe University, Institute of Health Science, Department of Pharmaceutical Toxicology PhD thesis, İstanbul.

Amanita Phalloides mushrooms containing α -AMA are responsible for the majority of fatal mushroom poisonings. EGF has a proliferative effect on cells of both mesodermal and ectodermal origin, especially keratinocytes and fibroblasts. According to experimental results EGF has a promising effect on liver toxicity. The purpose of this study was to investigate the effectiveness of EGF in both *in vitro* and *in vivo* models of α -AMA-induced hepatotoxicity and to compare this with Silibinin, a known antidote. The cytotoxicity and oxidative stress parameters were evaluated on HuH-7 hepatocellular carcinoma cell line. In this study, the effects of varying concentrations of SIL (12.5, 25, 50 μ M), EGF (1, 2, 4 μ M), and combined treatments of SIL and EGF were investigated. Malondialdehyde, glutathione, catalase and superoxide dismutase were observed. In the *in vivo* part of the project. EGF efficacy in α -AMA intoxication treatment were evaluated in an *in vivo* animal model by comparing with Silibinin. 36 Balb-C male mice were used and divided into six groups. The groups were treated with α -AMA and determined antidotes given for each group and observed for 48 hours. Alkaline phosphatase, ALT, AST, GGT levels were studied with specific ELISA test kits. Liver tissues were taken at the end of experiment and observed for Liver Histological Damage Score and active caspase-3 levels. EGF has decreased MDA more when compared to SIL treatment. The combination treatment of SIL and EGF indicated a MDA profile similar to high concentration of EGF. Glutathione, superoxide dismutase and catalase experiments indicated that AMA exposure slightly decreased every parameter and treatment groups ranged effectiveness in a dose dependent manner. *In vivo* study results showed that higher dose of EGF (EGF1500) significantly lower the liver function tests and observed better results on histomorphological analysis than α -AMA and EGF750 group both. EGF treatment showed promising results in α -AMA poisoning. Silibinin only and Silibinin and EGF combination have improved the liver function tests but cannot effectively treat the liver tissue. Therefore, it can be concluded that the use of EGF in high doses can be used to treat hepatotoxicity due to α -AMA poisoning.

Keywords: Amanitin, Silbinin, Epidermal growth factor, liver toxicity

ÖZET

Sinan K. (2023). Amatoksin Kaynaklı Hepatotoksisitede Epidermal Büyüme Faktörü Tedavisinin İncelenmesi. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü Farmasötik Toksikoloji Anabilim Dalı Doktora Tezi, İstanbul.

α -AMA içeren *Amanita Phalloides* mantarları, ölümcül mantar zehirlenmelerinin çoğundan sorumludur. EGF, hem mezodermal hem de ektodermal kökenli hücreler, özellikle keratinositler ve fibroblastlar üzerinde proliferatif bir etkiye sahiptir. Deneysel sonuçlara göre EGF'nin karaciğer toksisitesi üzerinde umut verici bir etkisi vardır. Bu çalışmanın amacı, α -AMA kaynaklı hepatotoksisitenin hem *in vitro* hem de *in vivo* modellerinde EGF'nin etkinliğini araştırmak ve bunu bilinen bir antidot olan Silibinin ile karşılaştırmaktır. Sitotoksisite ve oksidatif stres parametreleri HuH-7 hücre hattı üzerinde değerlendirildi. Bu çalışmada, değişen konsantrasyonlarda SIL (12.5, 25, 50 μ M), EGF (1, 2, 4 μ M) ve kombine SIL ve EGF tedavilerinin etkileri araştırıldı. Malondialdehit, glutatyon, katalaz ve süperoksit dismutaz düzeyleri ölçüldü. Projenin *in vivo* kısmında. α -AMA zehirlenme tedavisinde EGF etkinliği, Silibinin ile karşılaştırılarak bir *in vivo* hayvan modelinde değerlendirildi. 36 Balb-c erkek fare kullanıldı ve altı gruba ayrıldı. Gruplara α -AMA uygulandı ve her grup için belirlenen antidotlar uygulandı ve 48 saat gözlemlendi. Alkalın fosfataz, ALT, AST, GGT düzeyleri değerlendirildi. Deney sonunda karaciğer dokuları alındı ve Karaciğer Histolojik Hasar Skoru ve aktif kaspaz-3 seviyeleri değerlendirildi. EGF, MDA'yı SIL tedavisine göre daha fazla azaltmıştır. SIL ve EGF'nin kombinasyon tedavisi, yüksek EGF konsantrasyonuna benzer bir MDA profili gösterdi. Glutasyon, süper oksit dismutaz ve katalaz deneyleri, α -AMA maruziyetinin her parametreyi hafifçe azalttığını ve tedavi gruplarının doza bağlı bir şekilde etkinlik aralığında değiştiğini gösterdi. *In vivo* çalışma sonuçları, hem karaciğer fonksiyon testlerinde hem de histomorfolojik analizde daha yüksek EGF dozunun (EGF1500) α -AMA ve EGF750 grubuna göre anlamlı derecede düşük olduğunu göstermiştir. EGF tedavisi α -AMA zehirlenmesinde ümit verici sonuçlar verdi. Yalnızca Silibinin ve Silibinin ve EGF kombinasyonu, karaciğer fonksiyon testlerini iyileştirmiştir ancak karaciğer dokusunu etkili bir şekilde tedavi edemediği gözlenmiştir. Bu nedenle, yüksek dozlarda EGF kullanımının α -AMA zehirlenmesine bağlı hepatotoksisitenin tedavisinde kullanılabileceği sonucuna varılabilir.

Anahtar Kelimeler: Amanitin, Silbinin, Epidermal büyüme faktörü, karaciğer toksisitesi

1. INTRODUCTION AND PURPOSE

The subject of the thesis is the examination of epidermal growth factor (EGF) treatment in alpha-amanitin (α -AMA) induced acute hepatotoxicity. For this reason we aimed to examine the effectiveness of EGF treatment in α -AMA induced acute hepatotoxicity. The intervention appeared to amplify hepatic cells detoxification mechanisms which may ultimately attenuate hepatic stellate cells (HSC) activation and the onset of a fibrogenic program.

Morbidity and mortality caused by mushroom intoxications is an important global health problem. It is clear that approximately 100 species of mushroom are toxic (1). *Amanita Phalloides* mushrooms containing α -AMA are responsible for the majority of fatal mushroom poisonings. α -AMA is the primary toxin responsible for hepatotoxicity (2). After being absorbed by the gastrointestinal system and entering the systemic circulation, α -AMA passes into the hepatic sinusoidal membrane, its main target, with the organic anion transporter peptide (OATP)1B3. It then blocks mRNA synthesis and DNA transcription by inhibiting RNA polymerase II in the nucleus. Cell death therefore occurs as a result of deterioration of protein synthesis in cells with high turnover, such as hepatocytes, gastrointestinal system epithelial and hepatic tubule cells (3, 4).

EGF is a growth factor known for its proliferative and regenerative properties. In the context of cytotoxicity, EGF has been investigated as a potential protective agent against various toxins and hepatotoxic agents. Studies have shown that EGF treatment can enhance cell survival and tissue regeneration in liver injury models. EGF activates survival pathways, such as the PI3K/Akt pathway, which plays a crucial role in promoting cell survival and protecting against cytotoxic insults (5). The protective effects of EGF against liver injury have been demonstrated in models of hepatotoxicity induced by various agents, including acetaminophen and Carbon tetra chloride (CCl₄) (6, 7).

The purpose of this study was to investigate the early and late-term effectiveness of EGF in both *in vitro* and *in vivo* models of α -AMA-induced hepatotoxicity and to compare this with Silibinin, a known antidote.

2. LITERATURE REVIEW

2.1. Mushrooms

Mushrooms are a primitive group of living organisms without real organs such as roots, stems, leaves and flowers. There are approximately 1,500,000 species in this classification, including mold fungi, poisonous or non-poisonous mushrooms (8). According to some classifications, because it does not contain chlorophyll, it does not belong to the plant kingdom; nor it is not included in the animal kingdom because it cannot move and differs in cell structure. It is classified separately and included in the fungi world (9).

Mushrooms differ in structure, size and environment. They can be single or multi-celled and can live on land or in the sea. Since they have no chlorophyll, they cannot photosynthesize; Therefore, they are heterotrophs. Some species live parasites on other creatures, others rot on organic waste. Parasitic fungi are often a disease factor. Rotting fungi generally live on non-living vegetable tissues. Many edible mushrooms are studied in this group. Some fungi live together by forming algae and lichens. While it meets its nutrients from algae, it gives the water and minerals it takes from the soil to the algae thanks to its hyphae. Truffles known for their taste and economic value are examined in this group (8, 10, 11).

Fungal cells are larger than bacterial cells. Although there are no nuclei in the bacterial cell, there are one or more nuclei in the fungal cell. Chitin is found in the cell wall. Fungal cells form thread-shaped hyphae, and hyphae come together to form a reticulate mycelium. Mushrooms that secrete cellulose and lignin degrading enzymes from their mycelium absorb digested molecules (11).

Fungi can reproduce asexually or sexually. Asexual reproduction is with the help of spores, and generally fungi reproduce asexually. Terrestrial fungi reproduce with the help of endospores and exospores, and aquatic ones with the help of zoospores. Sexual reproduction of fungi involves the gathering of nearby gametes (9).

The pathological condition caused by some toxic compounds in the structure of cap mushrooms is called mushroom poisoning or mycetismus. Mushroom poisoning is more common, especially in the spring and autumn seasons, as it is consumed as a nutrient in

those with low socioeconomic status (2). There is no scientific value among the people, such as that poisonous mushrooms are remarkable, that they are not eaten by animals, that they turn black when a silver fork is inserted during cooking, and that mushrooms from certain regions are poisonous.

Due to the difficulty of diagnosis; Although data on the frequency of mushroom poisonings are limited, the World Health Organization reported that mushroom poisonings were responsible for 346,000 deaths between 2004 and 2014 (8). According to the 2014 Report of the American Association of Poison Control Centers, the rate of poisoning with mushrooms is approximately 0.3% (12). In the Western Black Sea region, the rate of mushroom poisoning was reported as 4.5% among the poisonings that ended in death in childhood in a four-year period. In a retrospective study of childhood mushroom poisonings in the Istanbul region, the rate of poisoning with *Amanita Phalloides* was 28% (1). In Turkey, 1.5-3.4% of admission to emergency services for poisoning are caused by mushroom poisoning (13).

Mushroom poisonings, according to clinical findings, are classified into three groups as early-onset (<6 hours), delayed-onset (6-24 hours), or late-onset (>1 day). Those with early onset; neurotoxic, gastrointestinal or allergic manifestations, delayed-onset causes toxic effects on the liver or kidney. Late-onset ones cause toxic effects on the kidney, neurotoxic effects, or rhabdomyolysis (14).

Although poisonous mushrooms give more than one toxic molecule, they are classified according to the main toxin they contain (Table 1) (15).

Table 1. Classification of the toxins of the mushrooms

Toxin Groups	Toxin	Mushroom Species
Orellanin	Orellanin	<i>Amanita smithiana</i> , <i>Cortinarius Splendens</i> , <i>Cortinarius Speciosissimus</i>
Mono Methyl Hydrazines (MMH)	Gyromitrin	<i>Gyromitra brunnea</i> , <i>Gyromitra esculenta</i> , <i>Helvella lacunose</i> , <i>Paxina species</i>
Disulfiram Like Reaction	Koprin	<i>Clitocybe claviceps</i> , <i>Coprinus atramentarius</i>
Muscarinic		<i>Boletus spp</i> , <i>Clitocybe Dealbata</i> , <i>Inocybe fastigiata</i>
Hallucinogen Indoles	Psilocin Psilocybin	<i>Conocybe cyanopus</i> , <i>Gymnopilus Validipes</i> , <i>Psilocyben cubensis</i>
Isoxazoles	Ibotenic acid Musimol	<i>Amanita muscaria</i> , <i>A. Pantherina</i> , <i>Panaeolus campanulatus</i>
Gastrointestinal System Irritants		<i>Boletus satanas</i> , <i>Lactarius torminosus</i> , <i>Russula emetica</i> ,
Cyclopeptides	Amatoxins Phallotoxins Virottoxins	<i>Amanita bisporigera</i> , <i>A. Phalloides</i> , <i>Galerina autumnalis</i> , <i>G. Marginata</i> , <i>Lepiota helveola</i>

Orellanin has the structure of 3,3',4,4'-tetrahydroxy-2,2'-bipyridyl. It is a colorless, polar and blue fluorescent compound (Figure 1).

**Figure 1.** *Amanita Smithiana* and Orellanin chemical structure (16)

It reduces glomerular filtration by acting on the kidneys. It causes Orellanus Syndrome. The lethal dose of fresh mushrooms in humans is estimated to be 100-200 g (21).

Monomethyl Hydrazines (Gyromitrin) is a methylhydrazine derivative. It is N-methyl N-formylhydrazone of acetaldehyde (Figure 2).

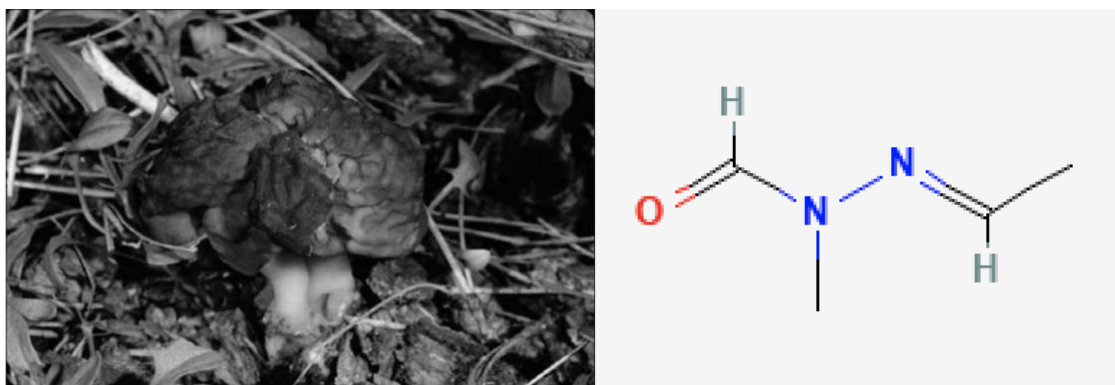


Figure 2. *Gyromitra Esculenta* and Gyromitrin chemical structure (17)

It is a volatile, colorless oily liquid at room temperature. Hydrazines, which are released by the breakdown of giromitrin and its homologues in stomach acid, cause necrosis in the liver. It causes Gyromitra syndrome. The lethal dose of giromitrin is 10-30 mg/kg in children and 20-50 mg/kg in adults (22).

Coprin is a colorless, crystalline compound in the structure of 5-N-(1hydroxycyclopropyl)-L-glutamine formed by the condensation of glutamine and cyclopropanone (Figure 3).

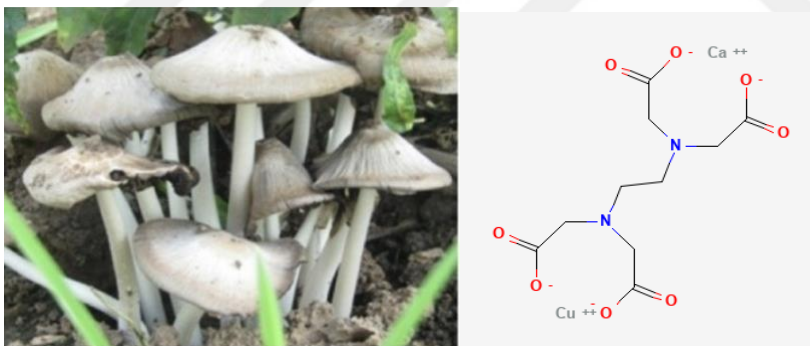


Figure 3. *Coprinus Atramentarius* and Coprin chemical structure (18)

It causes Coprinus syndrome. It inhibits the breakdown of alcohol in the body and stops it in the acetaldehyde phase. Normally, poisoning resulting from its consumption is minor, but occurs when alcohol and mushroom are consumed concomitantly. The mushroom has not, so far, caused fatal poisoning; nevertheless, the symptoms of poisoning, the antabuse effect, aroused the interest of pharmacologists and toxicologists, who sought to take advantage of a natural product that might be useful in the treatment of alcoholism (19).

Muscarine is a quaternary ammonium compound (Figure 4). Although Muscarine was first isolated and identified from *Amanita muscaria*, the actual muscarine content of *Amanita Muscaria* is very low (usually around 0.0003% of the fresh weight). *Inocybe* and *Clitocybe* spp has much higher Muscarine concentrations (1.6%). The poisoning presents classic parasympathetic stimulation with rapid onset (15 min–2 h). Resulted in chills, sweating and salivation. Muscarine's chemical structure is similar to acetylcholine. Muscarine affects the muscarinic acetylcholine receptor in the cholinergic nervous system, which does not excite nicotinic cholinergic receptors and cannot cross the blood–brain barrier to induce central cholinergic symptoms. The toxin is resistant to cooking and digestive system because of the molecular stability of the toxin, which enters the bloodstream through the lining of the intestine and then diffuses through the capillaries to the parasympathetic nervous system. Also, it cannot be hydrolyzed by plasma cholinesterase. For this reason, its peripheral cholinergic effects persist for several hours. Lethal dose of muscarine is around 180-300 mg (1,23).

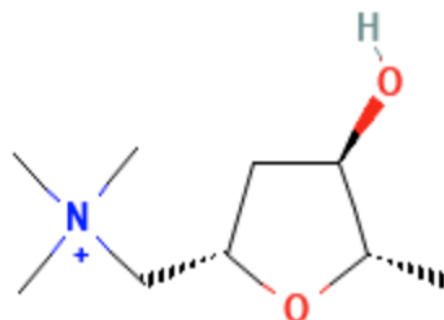


Figure 4. *Inocybe Rimosa* and molecular structure of Muscarine (20)

Hallucinogen Indoles are 4-substituted indole derivative compounds (Figure 5). It causes psilocybin syndrome. It acts on the central nervous system and causes hallucinations. Oral psilocybin is hydrolyzed to psilocin by the action of alkaline phosphatase in the body. Psilocybin is effective at very low doses. 4 mg causes mild intoxication and 6-20 mg causes significant psychotropic changes (1,24).

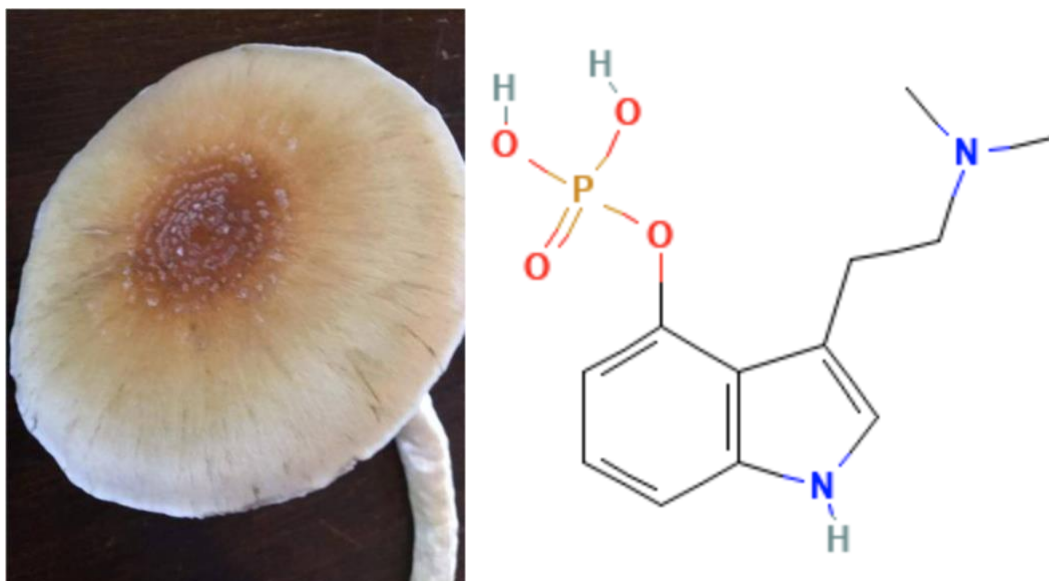


Figure 5. *Psilocybe Cubensis* and molecular structure of Psilocybin (21)

Ibotenic acid is a derivative of isoxazole. Melting at 145°C, this compound is colorless and crystalline. Mushroom contains only ibotenic acid, during drying, muscimol and muskazon are formed by decarboxylation of this compound or by photochemical reaction (Figure 6).

It causes Pantherina syndrome. Both compounds act on the central nervous system. muscimol is 5 times more active than ibotenic acid. Since its chemical structure is similar to gamma-aminobutyric acid (GABA), it can stimulate GABA receptors in central nervous system cells and therefore has a GABA-like effect (1). The toxic dose of muscimol is 109 µg/kg (1.25).

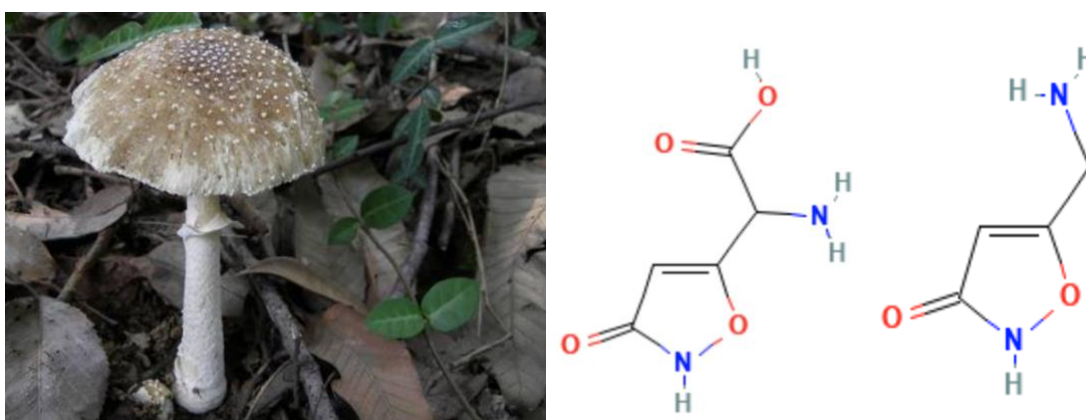


Figure 6. *Amanita Pantherina* and Ibotenic Acid [1] and Muscimol [2] chemical structure (22)

Mushrooms species that cause irritation in the gastrointestinal tract contain compounds of different structures. There is not enough information about the pharmacology of these compounds yet. Mushroom species that cause gastrointestinal syndrome or undesirable effects, or suspected toxicity, and compounds isolated from them are given (1,26).

- *Amanita citrina*: Bufotenine
- *Armillaria mellea*: Melleolid
- *Boletus satanas*: Xerocomic acid, Variegatic acid
- *Dermocybe sanguinea*: Dermocybin, Dermorubin
- *Hebeloma crustuliniforme*: Crustylinol
- *Hygrocybe species*: Hygro-aurins
- *Hypholoma fasciculare*: Fasciculol E, Nematolin
- *Lactarius helvus*: Stearylvelutinal, Nekatoron
- *Lyophyllum connatum*: Konnatin, Liophyllin
- *Omphalotus olearinus*: Illudin S

Cyclopeptides found in mushrooms are cyclic compounds composed of amino acids. Three groups of cyclopeptides were obtained from *Amanita* species.

Among these groups, only amatoxins are responsible for poisoning in humans. Fallotoxins and Virotoxins are not absorbed from the gastrointestinal tract.

Phallotoxins are bicyclic heptapeptides. They are colorless, crystalline and thermostable compounds soluble in water and methanol. Cytotoxic effects are prominent. Although it has been shown in studies to cause necrosis in the liver when administered parenterally, it does not have hepatotoxic effects by oral route. In animal experiments, it was found that they are not absorbed from the stomach. It has been understood that it has no role in poisoning (1).

Virotoxins are monocyclic heptapeptides. They are cytotoxic. They act like phallotoxins. It causes necrosis in the liver when given parenterally. When 2-3 mg/kg is given parenterally to white mice, it causes death within 2-5 hours (19).

Among the mushrooms containing cyclopeptide, *Amanita Phalloides* type mushrooms are the most common poisoning agents. The deaths due to mushroom

poisoning all over the world are mostly due to mushrooms containing toxic cyclopeptide. Diagnosis of this type of poisoning is very difficult and knowing the patient's history is life-saving (23).

2.2. *Amanita Phalloides*

Amanita Phalloides type mushrooms, also called "Death cap", belonging to the *Amanitaceae* family, were first described by the French botanist Sébastien Vaillant in 1727 (24).

Amanita Phalloides type mushrooms are grown in many countries of the world, including mainly European countries and Turkey, America, Asia, Australia and even the African continent in the temperate and humid climate zone (3). The most suitable breeding season for *Amanita Phalloides* is spring and autumn, and poisoning cases are most common in these seasons, grew especially in oaks, is seen as the most poisonous mushroom (25). Sometimes they can also be found in residential parks. They also live in the Belgrad Forests of Istanbul. This species is responsible for 95% of mushroom poisoning deaths in Turkey (26, 27). However, since the toxin causing the poisoning is stable, poisoning with such mushrooms can also be observed outside of spring months by eating canned mushrooms and dried mushrooms (28).

It has been reported that famous rulers in history such as Roman emperor Claudius', Russian Tsar Alexis, German emperor Charles (17th century) died due to poisoning with *Amanita Phalloides* mushroom (25).

Amanita Phalloides consist of 4 parts: cap, stem, annulus, volva. The surface of the cap is smooth, 6-12 cm wide. While it is greenish yellow in the young stage of the fungus, the color darkens to brown as it ages. The lamellae on the lower surface of the cap are independent from the stem, slightly radial striped and white. The stem is smooth, white in color and cylindrical about 6-12 cm high and slightly bulging downwards. It has a white membranous ring (annulus) at the top of the stem and a cup-shaped volva at the bottom of the stem. This ring and volva morphology is important in distinguishing *Amanita Phalloides* from other species. It is stated that the taste of the cap part is good and its smell is fruity (25). Physical properties can vary according to weather and soil

characteristics in different regions at different times of the year, and therefore it is difficult to distinguish from other fungal species (Figure 6) (29).

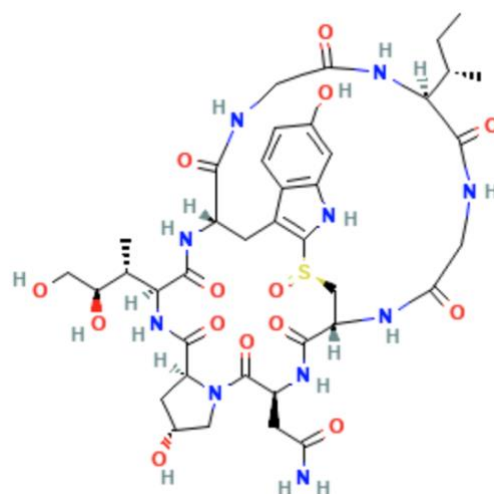


Figure 7. *Amanita Phalloides* (30)

Amanita Phalloides have about 15-20 cyclopeptide toxins. Among these toxins, the best known ones are Amatoxins (cyclic octapeptides), Phallotoxins (cyclic heptapeptides) and Virotoxins (cyclic heptapeptides) (23). Since all toxins contain tryptophan ring and indole ring, their chemical structures are similar, but their toxicity strengths are different from each other (31).

Virotoxins are monocyclic peptides. There are 5 types of virotoxins: alaviroidin, viroisin, deoxoviroisin, viroidin and deoxoviroidin (31). Their structural and biological activities are similar to phallotoxins and do not cause serious toxicity in humans after oral administration. In experimental studies, virotoxins have been shown to cause hemorrhagic liver necrosis when administered intraperitoneally to mice (32).

Phallotoxins are bicyclic heptapeptides. There are 7 types of phallotoxins: phalloidin, phalloin, profallin, phallicin, phallicin, phallacidin and phalisasin (31). Phallotoxins are toxins that show their effects quickly. Their oral absorption is low and their toxic effects on the liver have been shown only when administered parenterally (4). Phallotoxins are responsible for gastrointestinal symptoms such as diarrhea and vomiting occurring in the early stages in poisoning with *Amanita Phalloides* species (33).

Amatoxins are bicyclic octapeptides. There are 9 types of amatoxins: α -amanitin, β -amanitin, γ -amanitin, ϵ -amanitin, amanin, amaninamide, amanullin, amanullinic acid

and proamanullin. They differ from each other by the number of hydroxyl groups and by the diversity of amide carbonyl rings (Table 2) (31). Amanitins are colorless substances, soluble in water and methanol, and resistant to enzymes. Oral absorption is high. It is resistant to 250 °C and -25 °C. They cannot exceed the placenta, but are cytolytic. Amatoxins cause both liver, kidney and central nervous system damage and are the most toxic of the cyclopeptide toxins. α Amanitin (α -AMA) is the primary toxin responsible for liver toxicity (23).

Table 2. Chemical structure of amatoxins (31)

Compounds	Organic Compounds					LD ₅₀ (mg/kg)
	R1	R2	R3	R4	R5	
α -Amanitin	CH ₂ OH	OH	NH ₂	OH	OH	0.3
β -Amanitin	CH ₂ OH	OH	OH	OH	OH	0.5
γ -Amanitin	CH ₃	OH	NH ₂	OH	OH	0.2
ε -Amanitin	CH ₃	OH	OH	OH	OH	0.3
Amanin	CH ₂ OH	OH	OH	H	OH	0.5
Amaninamid	CH ₂ OH	OH	NH ₂	H	OH	0.3
Amanullin	CH ₃	H	NH ₂	OH	OH	20
Amanullic Acid	CH ₃	H	OH	OH	OH	20
Proamanullin	CH ₃	H	NH ₂	OH	H	20

2.2.1. α -Amanitin

2.2.1.1. Pharmacokinetic Properties

α -AMA is stable and resistant to high heat, cold, cooking, freezing, drying. It is easily absorbed from the gastrointestinal tract (28). It does not bind to plasma proteins and is soluble in water. After 90-120 minutes after oral ingestion, it is absorbed into the systemic circulation and reaches the target tissues, liver and kidney tissues, and is completely removed from the plasma at the end of 48 hours (34).

The toxin is not metabolized, a small part of it is excreted with bile. It participates in the enterohepatic circulation, which prolongs the exposure time of liver cells to the toxin (35). Most of the toxin is excreted in the urine through the kidneys. α -AMA can be detected in urine up to 3-4 days after oral ingestion (36).

2.2.1.2. Toxic Dose and Mechanism of Action

The lethal dose of α -AMA is 0.1 mg/kg for oral intake (4). A mushroom is approximately 60 g and contains 0.5-1.5 mg of α -AMA per gram. In this case, ingestion of even a single mushroom in an adult weighing 70 kg can cause lethal poisoning (37).

The target cell of α -AMA is primarily liver cells. Liver toxicity is the main cause of morbidity and mortality in poisoning due to α -AMA. However, gastrointestinal system epithelial cells and kidney proximal tubule cells are among the other cells affected (38, 39).

After α -AMA is absorbed from the gastrointestinal tract and enters the systemic circulation, it enters the cell with carrier proteins called OATP 1B3 located in the sinusoidal membrane of hepatocytes (40). Although many studies have been conducted on the mechanisms of intracellular toxicity, the most basic known mechanism is that α -AMA inhibits RNA polymerase II in the liver cell nucleus and prevents mRNA synthesis and DNA transcription (41). As a result of not synthesizing mRNA, protein synthesis stops in cells with high cellular turnover, such as liver cells, and liver cell necrosis develops (42).

2.3.2.2.A. Oxidative Stress

One of the most important mechanisms underlying liver toxicity is oxidative stress. Oxidative stress, which is defined as the deterioration of the balance between oxidants and antioxidants in favor of oxidants; It plays a role in the pathophysiology of many conditions such as cancer, neurological disorders, atherosclerosis, hypertension, ischemia-perfusion pathologies, diabetes, acute respiratory distress syndrome and asthma (43).

Reactive oxygen species (ROS) arise as a result of normal cellular metabolism in living organisms and contribute to physiological processes. However, at high concentrations, it causes changes in components of cells such as protein, lipid and DNA (44). Aerobic organisms are equipped with enzymatic and non-enzymatic antioxidant systems against the bad effects of ROS. However, in pathological conditions, these systems can be disabled and leave the cell vulnerable.

As an indicator of oxidative stress; MDA, the end product of lipid peroxidation, 8-hydroxy-2'-deoxyguanosine, which is an indicator of oxidative DNA damage, protein oxidation, antioxidant enzymes such as SOD, CAT, and the antioxidants such as glutathione was studied.

MDA is one of the end products of poly unsaturated fatty acid peroxidation in cells. The increase in free radicals may cause MDA overproduction. As mentioned before, MDA level is commonly known as a biomarker of oxidative stress and antioxidant status. PUFAs are also essential molecules in cellular membranes as a component of phospholipids, and peroxidation may occur under physiological conditions, which causes oxidation of these fatty acid chains. MDA forms as a result of this mechanism (43).

Catalase is found in different concentrations in mammalian cells. The amount of catalase in liver and kidney tissues was found to be higher than in heart and brain tissues. It contains Fe atom in the center of the molecule and converts 2 pieces of H_2O_2 to H_2O and O_2 by inducing when the cell is exposed to oxidative stress like SOD (45).

SOD converts two superoxide molecules into hydrogen peroxide and oxygen molecules (46). It has a rapid effect and it has been stated that it may be the first defense step against ROS (45).

Glutathione, which is a non-enzymatic antioxidant, takes part in the protection of cells against oxidative stress and radiation, the purification of toxic residues of drugs, and the metabolism of endogenic molecules such as locotriene and prostglandin. It interacts with free radicals and acts as an antioxidant as a substrate for glutathione peroxiadase (47).

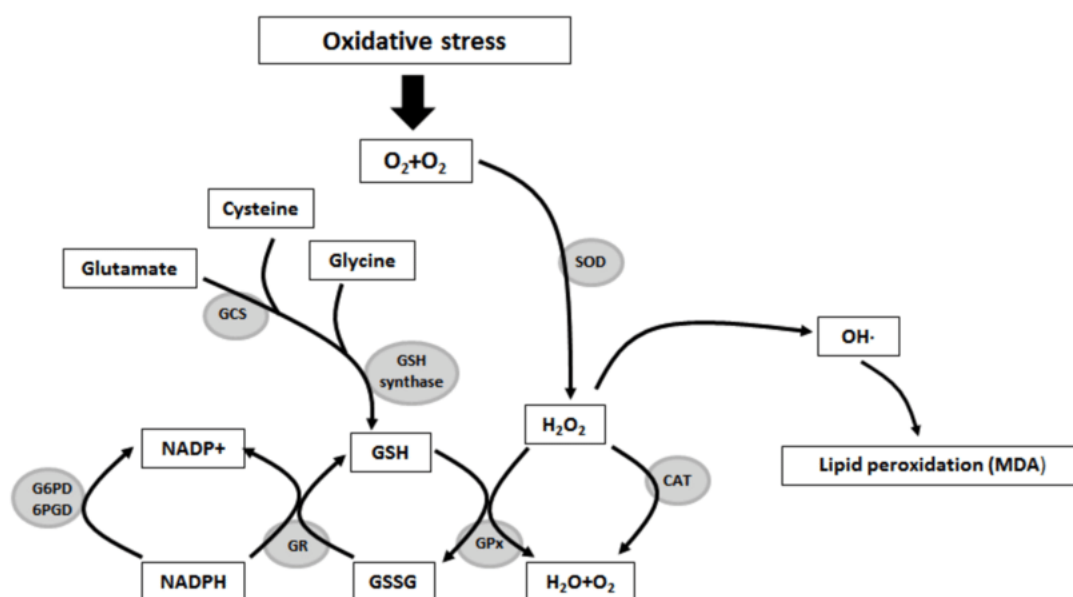


Figure 8. Oxidative stress pathway (48)

2.3.2.2.B.-Free Oxygen Radicals

ROS It is divided into two as free radicals and nonradicals.

Free radical: It is a high-energy molecule that carries 1 or more unpaired electrons in its molecule and gives reactivity to the molecule it encounters. Free radicals arise as a result of many reactions required for energy production in cell metabolism(44).

Nonradical: Molecules that are stable, have no unpaired electrons, and react more weakly with other molecules than free radicals.

Although there are many ROSs that are both free radical and nonradical, ROSs that cause important effects in the physiopathology of living things; superoxide anion, hydrogen peroxide and hydroxyl radicals (49).

Superoxide Anion: Superoxide anion; In aerobic organisms, it occurs as a result of reduction of molecular oxygen (O_2) by gaining an electron. Under physiological conditions, electrons are transported across the mitochondrial membrane via the electron transport chain, and oxygen is converted to water as a result of the transport. In the inner mitochondrial membrane, where the amount of electrons is high, superoxide anion is formed by the enzymes xanthine oxidase and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase. Superoxide anion is bactericidal in polymorphonuclear cells and macrophages.

Superoxides are converted to hydrogen peroxide by the enzyme superoxide dismutase. Then, with the Haber-Weiss and Fenton reactions, hydrogen peroxide is transformed into hydroxyl radicals through iron (Fe^{+2}) and copper (Cu^{+3}) (44).

Hydrogen peroxide: Hydrogen peroxide is formed by the granulocytic enzymes eosinophil peroxidase and myeloperoxidase. In the presence of Fe^{2+} or other transition metals (Fenton reaction) and superoxide radical (O^{2-}) (Haber-Weiss reaction), a strong radical hydroxyl radical ($\text{OH}^{\cdot}$) is formed. Unlike the superoxide radical, hydrogen peroxide dissolves in oil and can damage cellular membranes containing Fe^{2+} far from the place of origin (49).

Hydroxyl radicals: Hydroxyl radicals are the most potent radicals among ROS, which are formed from hydrogen peroxide and the reaction of metals with the Fenton and Haber-Weiss reaction. Because they are so strong, they cause damage to the cell by creating new radicals as a result of removing a proton from various molecules they encounter (50).

2.3.2.2.C. Hepatotoxicity

The liver is the primary organ where xenobiotics and toxins are metabolized, ie detoxified, and all substances taken through the gastrointestinal tract come to the liver via the portal vein (51). Many chemical and biological agents, including drugs, industrial substances, and herbal products that cause liver toxicity, are collectively called hepatotoxin. Despite the high regeneration capacity of the liver, hepatotoxins can cause necrosis or apoptosis in liver cells (52).

Liver toxicity is liver dysfunction and damage caused by drugs or xenobiotics. In clinical practice, liver toxicity is defined by a 3-fold or more increase in serum ALT level and/or a 2-fold or more increase in the upper limit of total bilirubin level (44). Liver toxicity, clinically may be asymptomatic or may progress to cholestasis, steatosis, acute liver necrosis and fulminant liver failure. The main reason for the variability of the clinical findings is due to the diversity of hepatotoxins and underlying pathophysiological mechanisms.

Liver toxicity occurs in three forms: necrosis, steatosis, and cholestasis. The most common type of liver toxicity is liver necrosis. Many drugs and toxic substances can

cause necrosis in the liver. In fact, acute liver necrosis is responsible for about half of the liver toxicities that develop. Cirrhosis, fibrosis, and veno-occlusive injury are also other types of liver toxicity (53).

2.3.2.2.D Acute Liver Necrosis

Acute liver necrosis is responsible for about half of liver toxicities. In acute liver necrosis; In the liver cells, a massive inflammatory reaction develops in response to the cellular content released by the rupture of the cell membrane. Histomorphologically, in the liver; inflammation, ballooned hepatocytes, sinusoidal enlargements are seen (54). In proportion to the extent of the necrosis, liver functions may be partially or completely lost. The most common inducers of acute liver necrosis are; paracetamol, fungi containing cyclopeptide such as *Amanita Phalloides*, volatile hydrocarbons such as carbon CCl₄ and isoniazid (53).

α -AMA has an important place among the toxic factors causing liver necrosis. The development of liver necrosis with α -AMA has been demonstrated in many experimental studies. The most known mechanism of α -AMA in the formation process of liver necrosis is binding to RNA polymerase II in hepatocytes and disruption of mRNA synthesis, which is indispensable for the synthesis of proteins required for the continuation of cell viability. Disruption of this synthesis causes direct cell death (3). Hepatocytes also initiate the process of necrosis or apoptosis by increasing the amount of ROS. In the experimental study of Dunder et al., the effect of α -AMA toxicity on oxidative stress biomarkers was investigated, and a significant increase was found in oxidative stress parameters such as super oxide dismutase (SOD) and malondialdehyde (MDA), especially as the dose of the toxin exposure increased (55). This increase was also found to be proportional to hepatic necrosis that developed at increasing doses due to α -AMA. *In vitro* studies hold that α -AMA induces apoptosis with its synergistic effect on Tumor Necrosis Factor- α (TNF- α) and creates oxidative stress due to increased ROS in liver toxicity due to α -AMA exposure (54). With the development of oxidative stress, an increase in SOD and MDA and a decrease in catalase levels are observed (56). The histomorphological findings of liver toxicity developed by α -AMA are fatty degeneration, centrilobular necrosis and hemorrhage (57). The necrosis and/or apoptosis process occurring at the cellular level causes the clinical findings of α -AMA to appear late.

2.3.2.2.E. Nephrotoxicity

Nephrotoxicity rarely seen in humans by AMA. It mostly seen in animal experiments or in kidney perfusion models. The liver most affected by the toxin due to first pass effect. Although a large amount of the toxin is excreted by the kidney, a small amount of toxin can be penetrated into the kidney cells because the kidney has no OATP system. early kidney damage is detected, this is an indicator of poor prognosis. Apart from being directly nephrotoxic, two other mechanisms have been described for the development of kidney damage. First, it is prerenal azotemia, which develops due to volume loss in the early stage of poisoning. The second mechanism is hepatorenal syndrome that occurs in the late period. Elevated BUN, creatinine and oliguria can be treated with fluid replacement in kidney damage due to hypovolemia. In the case of high serum creatinine that does not respond to fluid replacement on the third day or later, it suggests a direct toxic effect on the kidney. In amatoxin poisoning, metabolic acidosis does not occur before terminal circulatory failure, and this usually happens much later than 24 hours. An elevation of serum creatinine portends a bad prognosis at much lower levels than 300 $\mu\text{mol/l}$ (3.9 mg/dl) as seen by Kings College Criteria. Grade IV encephalopathy and coagulopathy are the excellent prognostic parameters in both intoxications (58).

2.2.2. Clinical Findings in α -Amanitin Poisoning

Amanita Phalloides type mushroom poisonings are clinically divided into four stages:

Latent Stage (Stage I): It is the asymptomatic stage. In the first 8-12 hours after the ingestion of the mushroom, no symptoms are observed in the patient. It is also reported that this phase can vary between 6-40 hours (42, 59).

Gastrointestinal Stage (Stage II): In this stage, watery diarrhea, colic-style abdominal pain and vomiting are observed. Symptoms begin as early as 6-24 hours after oral ingestion. Diarrhea may be bloody and mucous. This condition may be confused with viral gastroenteritis if mushroom poisoning is not suspected. In the laboratory, liver enzymes are normal, but electrolyte abnormalities secondary to severe diarrhea

(hypokalaemia, metabolic acidosis), acute renal failure, hypovolemic shock due to dehydration may occur.

Cytotoxic stage (Stage III): In this stage, the gastroenteritis clinic observed in patients improves, and the biochemical and clinical signs of liver toxicity become evident. Liver enzymes (AST, ALT), 24-36. starts to rise between hours and about 60-72 hours reaches its highest level. Hyperbilirubinemia, prolongation of prothrombin time, and disseminated intravascular coagulopathy findings are also observed in the laboratory (60).

Fulminant stage (Stage IV): In this stage, approximately 2-4 days after the mushroom is eaten, liver and kidney failure develop. Death in liver cells impairs liver blood flow. Due to hepatic failure, hypoglycemia, coagulopathy, encephalopathy, seizure, respiratory failure, multi-organ failure and death occur within 7-15 days (61).

2.2.3. Diagnosis in α -Amanitin Poisoning

In the diagnosis of poisoning with *Amanita Phalloides* type mushrooms; Having a history of eating wild mushrooms and the time of onset of the findings suggest poisoning. However, in poisoning with *Amanita Phalloides* fungi, the initial nausea, vomiting, abdominal pain and diarrhea are not specific to *Amanita Phalloides* poisonings and should be differentiated from gastroenteritis mostly due to bacteria, viruses or non-toxic fungi. Although the onset of the findings, especially within the first 6 hours following the mushroom ingestion, deviates from the diagnosis of poisoning with *Amanita Phalloides* type mushrooms, it cannot completely rule out the toxic mushroom. Because wild-collected mushrooms, even a single toxic mushroom in it, can cause fatal poisoning to the person who eats it. For this reason, various qualitative and quantitative methods that can be used in the clinic are being developed for the early and rapid diagnosis of the toxin in body fluids. Although not used routinely, the toxin of *Amanita Phalloides* species, which is toxic to the liver, is α -AMA; detectable in plasma or serum for up to 30 hours and in urine for up to 72 hours (35, 61).

Methods such as radioimmunoassay (RIA), enzyme-labeled immunosorbent assay (ELISA), capillary electrophoresis (CZE) and ultraviolet combined liquid chromatography (LC) for toxin detection in biological fluids cannot be used routinely in clinical applications due to false positive rate and impractical laboratory procedures.

However, liquid mass spectrophotometry (LC-MS/MS) method is a candidate for use in clinical practice, both qualitative and quantitative, in the early diagnosis of α -AMA with its high sensitivity and specificity rate (32). However, there is currently no routine diagnostic test in clinical use. It is often not possible to evaluate the fungus by a mycologist or to measure toxins from blood or urine. For these reasons, the differential diagnosis of mushroom poisoning is often based on clinical findings (25).

Edible mushrooms and poisonous mushrooms do not have definite distinguishing features. The methods used by the public to distinguish poisonous mushrooms such as their attractive appearance, their darkening when a silver fork is inserted during cooking, their inability to be eaten by wild animals, their blue insides when they are plucked, and the poisonousness of mushrooms grown in certain regions have no scientific value (32).

In patients with a history of picking mushrooms from nature and with delayed clinical findings such as nausea, vomiting and diarrhea, poisoning with *Amanita Phalloides* type mushrooms should be suspected and liver and kidney function tests should be checked from the blood taken from the patients as soon as they come, and the tests should be repeated at 24 and 48 hours (61).

2.2.4. Treatments in α -Amanitin Poisoning

In order to reduce the absorption of α -AMA from the gastrointestinal tract in poisoning with *Amanita Phalloides* type mushrooms, gastric lavage and activated charcoal application, which are among the decontamination methods, are recommended within the first 1 hour. However, since the toxin enters the enterohepatic circulation, repeated doses of activated charcoal also have a place in the treatment (38).

As an antidote, the application of Silibinin, which prevents the uptake of the toxin by the liver cell, is also included in high-dose crystalline penicillin treatment protocols (61).

In addition, ethanol, cimetidine, N-acetylcysteine (NAC), thioctic acid, and steroids are also potential treatment options (62). However, especially the results of cell culture studies report that early treatment should be initiated in order to prevent liver cell damage, since the toxin is retained by cells at an early stage (63, 64).

Silibinin is an antidote used in *Amanita Phalloides* mushroom poisoning. Silibinin inhibits the uptake of α -AMA by the liver cell by binding to its binding site on the liver cell, and also prevents the enterohepatic circulation of the toxin (65). It also increases glutathione stores, reduces leukocyte migration in the inflamed area and has an anti-inflammatory effect (63, 66). 20-50 mg/kg/day i.v. It is recommended to be applied for 48-96 hours by the route.

Penicillin G inhibits hepatic cell uptake of α -AMA as an OATP substrate at high doses and at high blood concentrations. In poisoning due to α -AMA, daily 300.000-1.000.000 U/kg i.v. It is recommended to be administered by infusion. It has been reported that high-dose beta-lactams can also prevent hepatic damage by inhibiting DNA polymerase alpha by reducing eukaryotic cell proliferation and GABA production (67).

NAC is a glutathione donor. In poisoning with *Amanita Phalloides* species, it can be administered with an infusion of 150 mg/kg in the first 60 minutes, followed by an infusion of 50 mg/kg for 4 hours, and an infusion of 6.25 mg/kg/hour until the patient's clinical recovery. However, there is not enough evidence about its effectiveness (68).

Thioctic acid has been used in various studies in the treatment of mushroom poisonings such as *Amanita Phalloides* with its antioxidant effect, which is manifested by the prevention of lipid peroxidation. A 150 mg/kg infusion is recommended in the first hour after poisoning, followed by 50 mg over 4 hours. Afterwards, it is administered at a dose of 50-150 mg every 6 hours. Although it has been shown to reduce mortality in various studies, there is insufficient evidence for their use in poisoning with this type of mushroom, and the fact that it causes hypoglycemia limits its use (69).

Methods such as hemodialysis, hemoperfusion, plasma exchange are very common procedures for toxin removal, and there are centers that still use these methods today. It has been reported that hemoperfusion and hemodialysis performed within the first 24 hours after mushroom ingestion may help to remove the toxin (70-72). However, the results about the effectiveness of these methods are controversial. Because there was no clinical improvement in patients who underwent these procedures, and no toxins were found in the dialysis fluids of these patients (73). The failure of these treatment modalities has been reported to be due to rapid tissue uptake of amatoxins and low plasma

concentrations. However, it has been reported that hemoperfusion may be effective in preventing cerebral edema due to fulminant liver failure (67).

Liver transplantation should be planned if all treatment options are taken into account, and if the patient still does not achieve a complete clinical recovery (74). However, it is very difficult to decide on liver transplantation in the early period. Ganzert et al.(75) retrospectively examined patients with amatoxin poisoning and showed that these patients were 3–10 years old. reported that daily prothrombin time and serum creatinine levels are predictive in terms of prognosis and death, prothrombin time and serum creatinine level are more sensitive and specific than all other parameters (including ALT and bilirubin levels), and these results may be effective in making the decision for liver transplantation.

2.2.5. α -Amanitin in Cell Culture Studies

In hepatocyte cell cultures, cell viability was decreased 24-48 hours after exposure to α -AMA, and it was reported that α -AMA was uptake by hepatocytes within 12 hours in hepatocyte cell cultures (76, 77). In cell culture studies, it has been shown that Silibinin, N-acetylcysteine and benzylpenicillin cause an increase in hepatocyte cell viability, while rifampicin and ceftazidime do not increase cell viability (76).

It has been reported that exposure to α -AMA for 12 hours in primary canine hepatocyte cell cultures causes a decrease in cell viability proportional to α -AMA concentration and exposure time. In another study, it was shown that apoptosis has a role in liver toxicity due to α -AMA in human liver cell culture and that apoptosis is caspase-3 dependent, which is one of the caspase enzymes that play an important role in programmed cell death and is considered an apoptotic marker. Although it has been reported that apoptosis is particularly evident after 6 hours of exposure, it has been shown that apoptosis and necrosis occur simultaneously in dog liver cell cultures in a manner proportional to the exposure time and concentration to α -AMA (39, 63).

In studies with cell culture, it has been reported that α -AMA increases lipid peroxidation, SOD level and decreases catalase level. In another study, positive effects of Silibinin on lipid peroxidation were observed, while a similar effect was not observed in benzylpenicillin. It has been shown that NAC and benzylpenicillin increase the number of living cells and reduce apoptosis in human liver cell culture (66). Therefore, in cell

culture, liver cell death is observed with α -AMA and it is effective when applied in the early period in various treatments.

2.3. Epidermal Growth Factor (EGF)

EGF was first developed in 1962 by Dr. It was isolated from male mouse submandibular salivary gland by Stanley Cohen (78). Cohen, while trying to isolate nerve growth factor (NGF) in the male mouse submandibular salivary gland, observed that the extract obtained from these glands causes premature eyelid opening and early teething when injected into newborn mice. He named this substance epidermal growth factor (78).

2.3.1. Biochemical Properties of EGF

EGF is a single-chain polypeptide with a molecular weight of 6,000 daltons, consisting of 53 amino acids without lysine, phenylalanine and alanine in its amino acid sequence.

Its isoelectric point is 4.5. Its solubility in water is very high. It is soluble in methanol and ethylene glycol. Mouse EGF has a molecular weight of 6041 Da and an isoelectric point of 4.6. Its biological activity is stable in boiling water. However, if it is heated in dilute acid or alkali, its biological activity will be damaged. Human and mouse EGFs with similar chemical properties show the same biological activities (79). Three of the molecules end with the -NH₂ group and the other three with the -COOH group. The polypeptide has six cysteine radicals and contains three disulfide bonds. These disulfide bonds are essential for the biological activity of the molecule. The precursor of EGF contains 1217 amino acids. These numbers are 24 times greater than mature EGF and are called prepro EGF (78).

2.3.2. Receptors of EGF

Most cells have receptors for EGF. The most numerous receptors are found on epithelial cells, but there are also receptors on endothelial cells, fibroblasts, and smooth muscle cells. It has the feature of stimulating angiogenesis and collagenase activity. It is found in biological fluids such as plasma, milk, urine, saliva. EGF, like all other growth factors, binds high affinity and low-capacity receptors to the respective cell surface. The activity within the EGF receptor is tyrosine kinase activity against EGF binding. The

kinase domain of the EGF receptor phosphorylates the EGF receptor itself. This is also called autophosphorylation. This process is also valid for other proteins in the signal transduction step. After aggregation and phosphorylation occur by combining with the EGF receptor, the EGF receptor passes into the cytoplasm together. There they combine with lysosomes to form receptorosomes and are degraded (78).

2.3.3. Biological Properties of EGF

EGF has a proliferative effect on cells of both mesodermal and ectodermal origin, especially keratinocytes and fibroblasts. EGF increases the granulation tissue, collagen and glycosaminoglycan levels, resulting in accelerated epithelialization and increased wound tension strength. Submaxillary salivary gland extracts, in which EGF is abundant, show an accelerating effect on wound healing (80). EGF is 0.5% of the total gland protein in the male mouse. Gregory et al. showed that human urine inhibits gastric acid secretion in dogs and named the active substance they isolated as Urogastrone. Urogastrone was named human EGF because of the similarity of this isolated substance to mouse EGF (78). EGF is synthesized in mouse submaxillary salivary gland and stored in tubular duct cells. However, the absence of any change in plasma EGF level after removal of the submaxillary salivary gland in mice suggests that EGF is synthesized elsewhere in the organism. First, Brunner's glands in the intestines synthesize and secrete significant amounts of EGF. EGF is found in the highest amount in the submaxillary salivary gland. Then, duodenum, Brunner's glands, thyroid, pancreas, adrenal gland, ovary, parotid gland, liver, esophagus, stomach, small intestine, colon, heart, kidney, prostate, skeletal muscle, smooth muscle, lungs, thymus gland can be counted among these. In addition to these, EGF is found in urine, gastric juice, saliva, bile, prostatic and seminal fluid, sweat, tears, amniotic fluid and breast milk, plasma and serum (80). EGF is mitogenic for many cells of mesodermal and ectodermal origin. Therefore, it is said that EGF is associated with cancer due to its effect on neoplastic growth (80). EGF has the feature of increasing ion uptake, glycolysis, DNA and RNA synthesis in cells where it is active. EGF plays a role in the development of the organism by affecting differentiation and growth. EGF enhances growth, proliferation and differentiation in various epithelial cell cultures. Peptide growth factors such as EGF stimulate mitogenesis of keratinocytes and fibroblasts, migration of keratinocytes, and tissue and granulation development. It has

been found that it accelerates epithelial regeneration in pigs and corneal epithelialization in rabbits (78).

EGF is a cytokine and stimulates tissue differentiation and organogenesis. Neonatal causes tooth eruption and premature opening of the eyelids in rodents, initiates lung maturation in fetal rats, sheep and rabbits, aids palate development in organ cultures, stimulates gastrointestinal, liver and pancreatic maturation, aids thyroid and adrenal gland development, mammary gland development and stimulates wound healing, inhibits gastric acid secretion, stimulates pituitary hormone secretion including growth hormone (GH), prolactin (PRL) and adrenocorticotrophic hormone (ACTH), stimulates placental lactogen and chorionic gonadotropin secretion from placental tissue. It plays a role in embryonic testicle development. While it plays a role in male reproductive function by stimulating the meiotic phase of spermatogenesis, it controls spermatogenesis through the activation of sertoli and leydig cells (78).

2.3.4. The Role of EGF in Wound Healing

In recent studies, both in the field of general medicine and in oral surgery, it has been shown that topically or systemically applied EGF has positive effects on wound healing. It has been experimentally shown in many studies that submaxillary salivary gland extracts, in which EGF is abundant, have an accelerating effect on wound healing(80). The most important known effect of EGF is to accelerate wound healing. It is known to accelerate wound healing by stimulating the production of proteins and the migration of epithelial cells. EGF plays an important role in wound healing by increasing the growth of fibroblasts and migration of keratinocytes (80). The effect of EGF on wound healing goes through three processes: inflammatory stage, fibroblastic stage and remodeling stage. EGF, a mitogenic polypeptide, begins to act on wound healing at the end of the inflammation phase and is known to induce fibroblastic formation, as well as stimulate the formation and epithelialization of granulation tissue. In addition, EGF initiates DNA synthesis and cell production and activates protein synthesis (78).

In the literature, a microemulsion formulation of EGF has been developed and its effect on gastric ulcer wounds in rats has been investigated, and it has been reported that this formulation has a positive effect on gastric ulcer wounds (81). EGF is known to be a cytoprotective and trophic agent for the gastrointestinal mucosa. EGF stops gastric acid

secretion and stimulates the proliferation of gastric mucosal cells. It has been shown that the area and number of ulceration in the stomach increase after sialoadenectomy (81).

In addition, it acts by reducing bacterial translocation in ischemia-reperfusion injury in the gastrointestinal tract (82). It also has a role in the repair and protection of the esophageal mucosa (83). In the study of Celebi et al., a polyacrylic acid gel formulation containing EGF was prepared and applied to 1 cm long skin incisions created on the mouse back. Polyacrylic acid has been preferred due to its adhesion to the mucosa and tissues and not being irritant. As a result of the examinations performed on the 7th and 15th days, it was observed that the tensile strength of the wounds in which EGF-polyacrylic acid gel formulation was applied was higher than the wounds in which EGF-containing saline was applied (81). In another study investigating the effect of EGF on chronic wounds, Silvaden containing EGF was applied topically in ulcer wounds caused by various diseases such as diabetes mellitus and rheumatoid arthritis, and it was reported that it had positive effects on healing (84). In a study investigating the effect of EGF in the healing of corneal alkaline burns in mice, it was stated that when EGF was applied to the wound area in various dosage forms, a significant decrease in the diameter of the injured area occurred compared to the control group (85). Jimenez et al. investigated the effect of EGF on the esophageal mucosa and concluded that EGF was effective in the proliferation of esophageal epithelial cells (86). Seatsuz et al. administered EGF and CAPE to rats with alkaline burns. As a result, they saw that EGF had a curative effect on the esophagus (87). In the study of Berthet et al. in which epidermal growth factor and interferon γ were administered intraperitoneally in rats, a significant decrease in esophageal thickness was observed in the group in which intraperitoneal epidermal growth factor and interferon γ were given together after esophageal burn was created (88).

2.3.5. The Role of EGF Treatment in CCl₄ Induced Hepatotoxicity

CCl₄ is a substance that causes death by disrupting the cell membrane structure and intracellular balance by forming lipid peroxidation in liver cells (89).

Administration of EGF at the lower dose (500 pg/kg) did not reduce CCl₄-induced hepatic injury, as assessed by histology. Enzyme levels were lower than those seen in animals given CCl₄ without EGF. In contrast, rats pre-treated with the higher dose of EGF (750 pg/kg) showed minimal changes in histology, with only occasional scattered

individual necrotic hepatocytes. Serum ALT levels were significantly lower than those seen in animals given CCl₄ without EGF, and were only about 50% higher than levels seen in animals not given CCl₄. MDA levels were not significantly different to those seen in animals not given CCl₄ (90).



3.MATERIALS AND METHOD

3.1. *In Vitro* Study Method

3.1.1. Cytotoxicity Assay

The cytotoxicity of substances was evaluated on HuH-7 hepatocellular carcinoma cell line which was obtained from American Type Culture Collection (ATCC, USA). The HuH-7 cells were cultured in RPMI 1640 medium (Gibco, NY, USA) supplemented with 10% Fetal Bovine Serum) (FBS and 1% penicillin (10.000 units/mL) and streptomycin (10.000 µg/mL) (Gibco, NY, USA), 1% MEM non-essential aminoacids (Gibco, NY, USA) and 1% L-Glutamine (Gibco, NY, USA) at 37°C under a humidified atmosphere of 5% CO₂. Complete cell culture media can be stated at Table 3.

Table 3. Complete cell culture media

Complete medium	
RPMI 1640 medium	46.5 mL
FBS	2.5 mL
Antibiotics (Penicillin+ Streptomycin)	0.5 mL
MEM non-essential aminoacids	0.5 mL
L-Glutamine	0.5 mL
<u>Final volume:</u>	50 mL

For evaluation of cytotoxicity of α -AMA, EGF and SIL, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay was used. 1.2×10^4 cells/well Plated HuH-7 cells were treated with substances with various concentrations. After 24 hours incubation, the cell culture medium was removed. MTT which was aliquoted as 5 mg/mL was diluted to 0.5 mg/mL. Then, MTT assay reagent was added to wells and cells were incubated for an additional 2 h at 37°C. After 2 hours incubation, cell culture medium was discarded and 100 µL of isopropanol was applied to wells to dissolve formazan crystals. The absorbance was measured at 570 nm wavelength by an ELISA microplate reader (Thermo Multiskan Spectrum, Finland). The percentage of cell viability was calculated by using the following equation:

$$Viability\% = (Absorbance\ treatment\ group) / (Absorbance\ control) \times 100\%$$

Concentrations indicating a viability under 70% were considered cytotoxic and non-cytotoxic doses were used in further experiments. Each experiment was performed in triplicates.

3.1.2. Oxidative Stress Parameters Assay

The HuH-7 cell lines 7×10^5 cells/well were cultured in six-well plates under standard conditions at a temperature of 37°C with 5% CO_2 for a period of 24 hours to achieve an 80-90% confluent cellular layer. Subsequently, the cells underwent treatment with α -AMA (10 μM), as per experimental protocol.

In this study, the effects of varying concentrations of SIL (12.5, 25, 50 μM), EGF (1, 2, 4 μM), and combined treatments of SIL and EGF were investigated. After being exposed, the cells underwent trypsinization and centrifugation. After this process, the residual cell pellet was rinsed with PBS. Then the pellet was subjected to centrifugation at 1000 g at a temperature of 4°C for 10 minutes. After subjecting the cell suspension to, a cell pellet was obtained.

The samples were transferred to Eppendorf tubes with a volume capacity of 1.5mL using 1 mL of phosphate-buffered saline (PBS). The cells were subjected to homogenization utilizing a tissue homogenizer, with rotational speeds gradually increasing from 30.000 rpm to 75.000 rpm in 30-second intervals. The cells that were fully lysed were subjected to centrifugation at a rate of 14.000 rotations per minute at 4°C for a duration of 15 minutes, following which the preservation of lysate at -20°C for subsequent assessment of protein content, MDA level, and oxidative stress parameters.

3.1.2.1. Malondialdehyde Determination

For MDA analysis, thiobarbituric acid (TBA), a chromogenic compound, was utilized with slight modifications of the methods defined by Buege et al. and Daraei et al. (91, 92). This method is based on the pink coloration of TBA when it reacts with MDA in an acidic medium at the appropriate temperature.

In summary, 200 μL of TBA mix (1x) containing TBA, trichloroacetic acid, HCl, and butylhydroxytoluene (BHT) was vortexed with 100 μL of cell lysate or a standard solution (tetramethoxypropane) in 1.5 mL Eppendorf tubes. The mixture was then incubated in a hot water bath for 30 minutes at 95°C . After cooling for 5 minutes, 100 μL of the pink-colored chromophore from each group was transferred to a 96-well plate and the absorbance was read at a wavelength of 540 nm using a UV spectrophotometer. Each measurement was performed in triplicate. The results were expressed as nmol/g protein.

Determination of Protein Content

The method described by Lowry et al. (93) with minor modifications was used to determine the protein content in the samples. In summary, 50 μL of cell lysate was mixed with 150 μL of a copper reactive solution containing sodium carbonate, sodium potassium tartarate, and copper sulfate in a 96-well plate. The mixture was then incubated for 10 minutes at room temperature.

Following the incubation, 50 μL of folin-phenol reagent was added to each well and incubated at 50°C for 10 minutes. The absorbance was measured at 690 nm using a spectrophotometer (Thermo Multiskan Spectrum, Finland). Bovine serum albumin (BSA) at various concentrations (1 mg/mL) was used as a standard for determining the protein content. The results were expressed in $\mu\text{g/mL}$.

3.1.2.2. Determination of Glutathione Levels

The GSH level was determined in cell lysates prepared using a modified version of the method developed by Sedlak et al.(94). To begin, 50 μL of cell lysate was mixed with 10 μL of DTNB solution and 150 μL of buffer solution in a 96-well plate. The mixture was then incubated at 37°C in darkness for 30 minutes. After the incubation period, the absorbance of the resulting, yellow-colored chromophore was measured at a wavelength of 412 nm using a UV spectrophotometer (Thermo Multiskan Spectrum, Finland). PBS was used as a blank. Each measurement was performed in triplicate. The GSH level results were expressed as $\mu\text{mol/g}$ protein. The solutions used for measuring the GSH level are described at Table 4.

Table 4. The solutions used for measuring GSH levels

Buffer Solution (pH 8.2):		
dH ₂ O		50 mL
Trizma Base		1.51 g
EDTA		3.65 mg
DTNB Solution, 10μM (Ellman's Reagent):		
DTNB		1.35 mg
MeOH		340 μ L
GSH Stock Solution, 10 mM:		
GSH		3.07 mg
PBS		1 mL
GSH Standards		
GSH stock vol.	Diluent (PBS) vol.	Final GSH Concentration (mM)
200 μ L	800 μ L	2 mM
100 μ L	900 μ L	1 mM
50 μ L	950 μ L	0.5 mM
25 μ L	975 μ L	0.25 mM
12.5 μ L	987.5 μ L	0.125 mM

3.1.2.3. Determination of Catalase Levels

CAT activity was measured from previously prepared cell lysates by modification of a method performed by Hadwan et al. (95). This method is based on the correlation between carbonato-cobaltate (III) complex and catalase enzyme. Turn of hydrogen peroxide (H₂O₂) into molecular oxygen and water (H₂O) in a chemical reaction controlled by CAT was evaluated through absorbance of the carbonato-cobaltate (III) complex in 2 minutes. According to present method, decrease in color intensity associates with increased CAT activity. Briefly, 50 μ L of cell lysate is mixed with twice amount of 10 mM H₂O₂ in tubes, mixed with vortex and incubated in 37 °C for 2 min in Eppendorf tubes. Following, 600 μ L of working solution containing phosphate buffer (pH 7.4), sodium bicarbonate and cobalt (II) nitrate hexahydrate was added and mixed with vortex immediately. Reaction tubes were kept in dark for 10 min and 250 μ L of reaction for each group were transferred to 96-well plates. Distilled water was used as blank. Absorbances were recorded at 440 nm spectrophotometrically and results were expressed as U/ mg protein. For calculation of CAT activity, given volumetric amounts and formula were given at Table 5.

Table 5. Calculation of CAT Activity and volumetric amounts

Reagents	Test	Standard	Blank
Cell lysate	50 µL	-	-
Blank (dH ₂ O)	-	50 µL	150 µL
H ₂ O ₂	100 µL	100 µL	-
Working solution	600 µL	600 µL	600 µL

According to the rate constant of a first-order reaction (k) equation by method of Hadwan et al. (95) was used to determine catalase activity, given below:

$$\text{Catalase Activity of test } kU = (2.303/t) \times \log (S_0 / S)$$

t: time, *S*₀ : absorbance of standard tube, *S*: absorbance of test tube

3.1.2.4. Determination of Superoxide Dismutase Levels

The SOD activity was determined using a modified version of the method developed by Marklund et al. (96). This method relies on the ability of intracellular SOD to inhibit the autooxidation of pyrogallol to pyrogallol-ortho-quinone in a reaction medium.

In summary, 10 µL of cell lysate prepared from confluent 6 well plates was incubated with 150 µL of a reaction mixture and 50 µL of EDTA in a 96-well plate. Then, 2 mM pyrogallol was added to each reaction mixture, and the changes in absorbance were immediately recorded for 450 seconds at 420 nm. Each measurement was taken at 30-second intervals for a total of 15 recordings. The first measurement taken after the addition of pyrogallol to the reaction medium was considered as time 0. Distilled water was used as a blank for all measurements.

The amount required to inhibit 50% of pyrogallol autooxidation was considered as 1 U of SOD activity. SOD activity measurements were performed in duplicate for each group. The results were expressed as U/mL. The calculation of the percentage inhibition of pyrogallol autooxidation was performed using the following formula:

$$\% \text{ Inhibition of pyrogallol autooxidation} = [(Absorbance \text{ control} - Absorbance \text{ sample}) / Absorbance \text{ control}] \times 100$$

The calculation of SOD activity was performed using the following formula:

$$SOD \text{ activity (U/mL)} = [(Absorbance \text{ control} - Absorbance \text{ sample}) / Absorbance \text{ control}] \times Total \text{ volume of reaction mixture} / Volume \text{ of sample (mL)}$$

3.2. *In Vivo* Study Method

In the *in vivo* part of the project. EGF efficacy in α -AMA intoxication treatment was evaluated in an *in vivo* animal model by comparing with Silibinin after ethical approval. Biochemical analysis and histopathological examinations were performed after the tissue blood and samples were taken.

Information about the experimental animal planned to be used in the *in vivo* study is given in Table 6. Mice fed a standard Mouse diet ad libitum.

Table 6. Information about the experimental animal planned to be used

Species	Breed	Gender	Weight	Count
Mice	BALB-c	Male	25–30 gram	36

36 male mice weighing between 25-30 grams, are randomly divided into groups. Experimental groups for acute toxicity study were formed according to Table 7.

Table 7. Acute toxicity model experimental groups

No	Acute Toxicity Groups (n= 6)	Definitions	Route
1	Control Group	This group will be administered intraperitoneal isotonic saline for control purposes.	IP
2	α -AMA	To create an acute poisoning model, 0.75 mg/kg α -Amanitin will be administered.	IP
3	AMA+EGF750	EGF 750 μ g and 0.75 mg/kg α -Amanitin will be administered to these groups.	IP
4	AMA+EGF1500	EGF 1500 μ g and 0.75 mg/kg α -Amanitin will be administered to these groups.	IP
5	AMA+SIL	0.75 mg/kg α -Amanitin and 5 mg/kg Silibinin will be administered to this group.	IP
6	AMA+SIL+EGF750	0.75 mg/kg α -Amanitin, 5 mg/kg Silibinin, and 750 μ g/kg EGF will be administered to this group.	IP

According to the literature review, the predicted dose was determined as 750 µg/kg for EGF. For the acute toxicity study, the animals were fasted for 12 hours, then 0.75 mg/kg α -AMA was administered, followed by EGF and Silibinin 6 hours later. Blood was collected from mice for biochemical analysis before and 48 hours after administration and were periodically observed at 6, 12, 18, 24 and 48 hours for signs of toxicity or death, and then decapitated at the end of 48 hours and blood samples and liver tissues were obtained (11,12).

Hour	0	6	12	18	24	48
α -AMA	0,75mg/kg	NS	NS	NS	NS	X
AMA +EGF750	0,75mg/kg	750µg/kg	NS	NS	NS	X
AMA +EGF1500	0,75mg/kg	1500µg/kg	NS	NS	NS	X
AMA +SIL	0,75mg/kg	5 mg/kg	5 mg/kg	5 mg/kg	5 mg/kg	X
AMA +SIL+EGF750	0,75mg/kg	750µg/kg + 5 mg/kg	5 mg/kg	5 mg/kg	5 mg/kg	X

Figure 9. Follow-up and Treatment Diagram of the Experiment Groups

For biochemical analysis, serum samples were made with alkaline phosphatase, ALT, AST, GGT specific ELISA test kits.

Samples of liver tissue were fixed in 10% buffered formaldehyde and embedded in paraffin. Then, 5 µm sections were taken from liver tissue samples and stained with Hematoxylin-Eosin (HE).

The sections taken were left in the oven at 60 ° C for 2 hours for deparaffinization process. Then the first was subjected to xylene for 20 minutes (in the oven) and the other two for 10 minutes in three different environments. Then, for the rehydration process, 2 changes were passed through absolute alcohol and alcohol series decreasing from 96% to 70%. Sections were stained with Hematoxylin for 10 minutes after rinsing with distilled water. After staining, sections washed in running water for 10 minutes to remove excess dye from the tissue were stained with Eosin dye for 2 minutes. After dyeing, sections

passed through 70%, 80%, 96% and 2 series of absolute alcohol sequences, respectively, were covered with three changes of xylene for 20 minutes each for transparency.

Liver tissue samples were examined for necroinflammation and apoptosis. Changes in liver tissue morphology;

In terms of congestion, necrosis, cytoplasmic vacuolization, eosinophilia, nuclear pyknosis, karyorrhexis and inflammatory cell density, the pathologist involved in the study was blindly evaluated under the light microscope.

Liver Histological Damage Score (LHDS) was used for evaluation.

0: No damage or minimal damage,

1: Slight damage,

2: Moderate damage,

3: It will be graded as severe damage.

Evaluation of Apoptosis Presence

Assessment of apoptosis done both by determining the number of apoptotic hepatocytes showing findings such as cytoplasmic eosinophilia and shrinkage, nuclear pyknosis and fragmentation at a large magnification (x400) in the area where the most intense apoptosis is detected in hematoxylin eosin-stained sections, and immunohistochemistry using antibodies against Caspase-3.

Active caspase-3 immunohistochemical staining method;

The sections were stained with caspase-3 staining method after waiting in a 60 °C oven for 24 hours. Mouse specific Cleaved Caspase-3 (Asp175) (5A1E) (Cell Signaling, MA, USA) antibody was used to demonstrate caspase-3 immunoreactivity. The lysine sections were deparaffinized with three exchanges of xylene. Afterwards, it was rehydrated with decreasing alcohol series and kept in distilled water for 5 minutes. In order to inhibit tissue endogenous peroxidase, 3% H₂O₂ will be applied for 10 minutes. It was washed twice for 5 minutes in distilled water. Preparations were washed with washing solution for 5 minutes. The sections were incubated with the blocking solution

at room temperature for 1 hour and then incubated with anti-caspase-3 antibody at + 4°C overnight without washing. The next day, the preparations were washed three times with washing solution for 5 minutes. Washed sections were incubated with biotinylated secondary antibody for 30 minutes. After the secondary antibody was bound with the enzyme-labeled (peroxidase) avidin-biotin complex (streptavidin), the antibody-biotin-avidin-peroxidase complex was made visible using 0.02% DAB. Floor painting was done with Mayers hematoxylin. Sections where dehydration process is performed in graded alcohols were closed with entellan after transparentization with xylol.

For immunohistochemical scoring, the degree of positive staining was assessed by semi-quantitative immunoreactivity scoring using an indicator chart from 0 to 3 in terms of both intensity and distribution (13).

- 0: No immunoreactivity,
- 1: Immunoreactivity is mild,
- 2: Immunoreactivity medium,
- 3: Immunoreactivity evident.

3.3. Statistical Analysis

Statistical analysis performed using SPSS version 25 and Jamovi version 2.3.21.0 using two-way ANOVA for group comparisons. Measurements were used to plot for regression and slope analysis, and group comparisons were carried out using one-way ANOVA. Tukey-Kramer multiple comparison tests were used to determine which group the difference originates from.

Analysis of data done with the help of Graphad Instat V2.05a- 1994. All data are presented as Mean±Standard error mean. $p < 0.05$ considered statistically significant.

4. RESULTS

4.1. *In Vitro* Study Results

4.1.1. Cytotoxicity

Cytotoxic effects of various concentrations of EGF, SIL and AMA on HuH-7 cell line has been shown in Figure 9.

For AMA, dose selection was conducted according to Kim et al. (97). In order to observe dose dependency, 20 and 40 micromolar of AMA has been taken into account. For future analysis, 10uM of AMA has been selected. According to Figure 9., SIL, EGF and their combination treatments were not cytotoxic and furthermore they have increased cell viability.

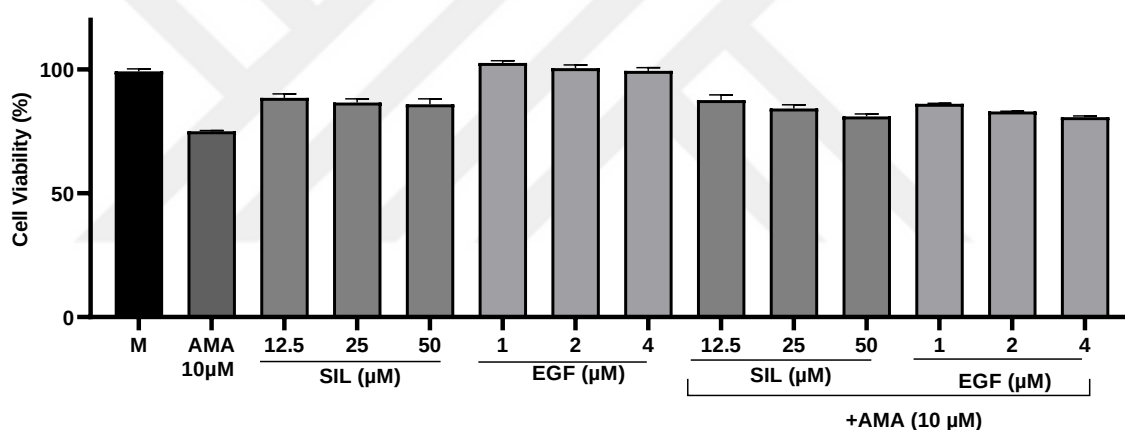


Figure 10. The effect of AMA, SIL and EGF in HuH-7 Cell Line

4.1.2. Oxidative Stress Parameters

In assessing oxidative stress parameters, SOD, CAT, GSH, MDA were taken into account. All experiments showed statistically significant difference between groups Games-Howell post hoc analysis performed to find the cause of difference in which group. Table 8 showed the results of all experiments.

Table 8. Oxidative stress parameters measurements

	Group	Mean±SD	p
SOD	M	0.3900±0.02828	< .001
	AMA 10 µm	0.0367±0.00206	
	EGF 1 µm+AMA 10 µm	0.0715±0.00919	
	EGF 2 µm+AMA 10 µm	0.1190±0.00990	
	EGF 4µm+AMA 10 µm	0.3840±0.00707	
	EGF 2 µm+ SIL 25 µm+ AMA 10 µm	0.2900±0.04101	
	SIL 12.5µm+ AMA 10 µm	0.0287±0.00898	
	SIL 25 µm+AMA 10 µm	0.0292±0.00686	
	SIL 50 µm + AMA 10 µm	0.0590±0.01131	
CAT	M	41.7416±2.02432	0.007
	AMA 10 µm	15.3515±2.17479	
	EGF 1 µm+AMA 10 µm	21.6443±2.72333	
	EGF 2 µm+AMA 10 µm	22.2201±1.53056	
	EGF 4µm+AMA 10 µm	32.3013±2.17181	
	EGF 2 µm+ SIL 25 µm+ AMA 10 µm	31.9060±0.44682	
	SIL 12.5µm+ AMA 10 µm	18.4109±3.51488	
	SIL 25 µm+AMA 10 µm	29.5379±2.20862	
	SIL 50 µm + AMA 10 µm	42.9531±1.34552	
GSH	M	538.1277±3.37850	< .001
	AMA 10 µm	446.8963±1.73298	
	EGF 1 µm+AMA 10 µm	786.9264±1.44622	
	EGF 2 µm+AMA 10 µm	804.7228±3.00681	
	EGF 4µm+AMA 10 µm	854.7947±4.41321	
	EGF 2 µm+ SIL 25 µm+ AMA 10 µm	780.4072±0.89632	
	SIL 12.5µm+ AMA 10 µm	595.9742±1.92426	
	SIL 25 µm+AMA 10 µm	615.6774±3.87301	
	SIL 50 µm + AMA 10 µm	682.9273±3.69067	
MDA	M	7.5068±0.59964	0.002
	AMA 10 µm	29.5537±1.01191	
	EGF 1 µm+AMA 10 µm	23.9223±1.82785	
	EGF 2 µm+AMA 10 µm	20.0066±3.17209	
	EGF 4µm+AMA 10 µm	16.5120±1.84068	
	EGF 2 µm+ SIL 25 µm+ AMA 10 µm	18.2119±3.11845	
	SIL 12.5µm+ AMA 10 µm	26.3398±3.02354	
	SIL 25 µm+AMA 10 µm	23.7945±0.83268	
	SIL 50 µm + AMA 10 µm	20.5422±0.16905	

In control SOD levels were stated as 0.39±0.03 and in AMA group SOD levels were decreased down to 0.04±0.01. ANOVA analysis showed statistically significant difference between groups ($p < 0.001$). Tukey post hoc analysis performed to find the cause of difference in which group. In EGF, SOD increased in a dose dependent manner. EGF 4 µm concentration indicated a similar profile to Medium ($p=1.00$). Especially in EGF 2 µm and EGF 4 µm concentrations showed statistically significant difference when compared to AMA ($p=0.023$, $p < 0.001$). In SIL similar to EGF, SOD increased in a dose

dependent manner but none of the concentration showed statistically significant change when compared to AMA. When SIL and EGF were considered, EGF has increased more when compared to SIL treatment. EGF 2 μm concentration showed statistically significant difference with SIL 12.5 μm and 25 μm concentrations ($p=0.013$, $p=0.014$). EGF at 4 μm concentration when compared to SIL 12.5 μm , 25 μm and 50 μm concentration ($p<0.001$). The combination treatment of SIL and EGF indicated a SOD profile similar to high concentration of EGF and showed statistically significant difference when compared to AMA (Table 9, Figure11).

In control CAT levels were stated as 41.74 ± 2.02 and in AMA group CAT levels were decreased down to 15.35 ± 2.17 . ANOVA analysis showed statistically significant difference between groups ($p=0.007$). Tukey post hoc analysis performed to find the cause of difference in which group. In EGF, CAT increased in a dose dependent manner. Only EGF 4 μm concentrations showed statistically significant difference when compared to AMA ($p<0.001$). In SIL similar to EGF, CAT increased in a dose dependent manner. SIL 50 μm concentration indicated a similar profile to Medium ($p=0.999$). Especially SIL 25 μm and 50 μm concentration showed statistically significant change when compared to AMA. When SIL and EGF were considered, SIL has increased more when compared to EGF treatment. SIL 50 μm concentration showed statistically significant difference with EGF 2 μm and EGF 4 μm concentrations ($p<0.001$). The combination treatment of SIL and EGF indicated a CAT profile similar to high concentration of EGF and showed statistically significant difference when compared to AMA (Table 10, Figure11).

Table 9. SOD levels post hoc analysis between groups

		AMA 10 µm	EGF 1 µm +AMA 10 µm	EGF 2 µm +AMA 10 µm	EGF 4µm +AMA 10 µm	EGF 2 µm+ SIL 25 µm+ AMA 10 µm	SIL 12.5µm +AMA 10 µm	SIL 25 µm +AMA 10 µm	SIL 50 µm +AMA 10 µm
M	p- value	< .001	< .001	< .001	1.000	0.007	< .001	< .001	< .001
AMA 10 µm	p- value	—	0.621	0.023	< .001	< .001	1.000	1.000	0.930
EGF 1 µm+AMA 10 µm	p- value		—	0.296	< .001	< .001	0.399	0.412	0.998
EGF 2 µm+AMA 10 µm	p- value			—	< .001	< .001	0.013	0.014	0.122
EGF 4µm+AMA 10 µm	p- value				—	0.010	< .001	< .001	< .001
EGF 2 µm+ SIL 25 µm+ AMA 10 µm	p- value					—	< .001	< .001	< .001
SIL 12.5µm+ AMA 10 µm	p- value						—	1.000	0.751
SIL 25 µm+AMA 10 µm	p- value							—	0.765

Table 10. CAT levels post hoc analysis between groups

		AMA 10 µm	EGF 1 µm +AMA 10 µm	EGF 2 µm +AMA 10 µm	EGF 4µm +AMA 10 µm	EGF 2 µm+ SIL 25 µm+ AMA 10 µm	SIL 12.5µm +AMA 10 µm	SIL 25 µm +AMA 10 µm	SIL 50 µm +AMA 10 µm
M	p- value	< .001	< .001	< .001	0.030	0.023	< .001	0.006	0.999
AMA 10 µm	p- value	—	0.208	0.147	< .001	< .001	0.870	0.002	< .001
EGF 1 µm+AMA 10 µm	p- value		—	1.000	0.014	0.018	0.837	0.078	< .001
EGF 2 µm+AMA 10 µm	p- value			—	0.020	0.026	0.708	0.111	< .001
EGF 4µm+AMA 10 µm	p- value				—	1.000	0.030	0.002	0.918
EGF 2 µm+ SIL 25 µm+ AMA 10 µm	p- value					—	0.003	0.962	0.011
SIL 12.5µm+ AMA 10 µm	p- value						—	0.011	< .001
SIL 25 µm+AMA 10 µm	p- value							—	0.003

In control GSH levels were stated as 538.13 ± 3.38 and in AMA group GSH levels were decreased down to 446.90 ± 1.73 . ANOVA analysis showed statistically significant difference between groups ($p < 0.001$). Tukey post hoc analysis performed to find the cause of difference in which group. In EGF, GSH increased in a dose dependent manner. All concentrations showed statistically significant difference when compared to AMA ($p < 0.001$). In SIL similar to EGF, GSH increased in a dose dependent manner at all concentrations showed statistically significant change when compared to AMA ($p < 0.001$). When SIL and EGF were considered, EGF has increased GSH more when compared to SIL treatment. All concentrations of EGF shows statistically significant difference when compared to SIL concentrations. The combination treatment of SIL and EGF indicated a GSH profile similar to high concentration of EGF and showed statistically significant difference when compared to AMA (Table 11, Figure11).

In control MDA levels were stated as 7.51 ± 0.60 and in AMA group MDA levels were increased up to 29.55 ± 1.01 . ANOVA analysis showed statistically significant difference between groups ($p = 0.002$). Tukey post hoc analysis performed to find the cause of difference in which group. In EGF, MDA decreased in a dose dependent manner. Especially in EGF 2 μm and EGF 4 μm concentrations showed statistically significant difference when compared to AMA ($p = 0.019$, $p = 0.002$). In SIL similar to EGF, MDA decreased in a dose dependent manner but only SIL at 50 μm concentration showed statistically significant change when compared to AMA. When SIL and EGF were considered, EGF has decreased MDA more when compared to SIL treatment. EGF at 4 μm concentration when compared to SIL 12.5 μm concentration. The combination treatment of SIL and EGF indicated a MDA profile similar to high concentration of EGF and showed statistically significant difference when compared to AMA (Table 12, Figure11).

Table 11. GSH levels post hoc analysis between groups

		AMA 10 μm	EGF 1 μm +AMA 10 μm	EGF 2 μm +AMA 10 μm	EGF 4 μm +AMA 10 μm	EGF 2 μm + SIL 25 μm + AMA 10 μm	SIL 12.5 μm +AMA 10 μm	SIL 25 μm +AMA 10 μm	SIL 50 μm +AMA 10 μm
M	p- value	< .001	< .001	< .001	< .001	< .001	< .001	< .001	< .001
AMA 10 μm	p- value	—	< .001	< .001	< .001	< .001	< .001	< .001	< .001
EGF 1 μm +AMA 10 μm	p- value		—	0.004	< .001	0.467	< .001	< .001	< .001
EGF 2 μm +AMA 10 μm	p- value			—	< .001	< .001	< .001	< .001	< .001
EGF 4 μm +AMA 10 μm	p- value				—	< .001	< .001	< .001	< .001
EGF 2 μm + SIL 25 μm + AMA 10 μm	p- value					—	< .001	< .001	< .001
SIL 12.5 μm + AMA 10 μm	p- value						—	0.002	< .001
SIL 25 μm +AMA 10 μm	p- value							—	< .001

Table 12. MDA levels post hoc analysis between groups

		AMA 10 µm	EGF 1 µm +AMA 10 µm	EGF 2 µm +AMA 10 µm	EGF 4µm +AMA 10 µm	EGF 2 µm+ SIL 25 µm+ AMA 10 µm	SIL 12.5µm +AMA 10 µm	SIL 25 µm +AMA 10 µm	SIL 50 µm +AMA 10 µm
M	p-value	< .001	< .001	0.003	0.028	0.009	< .001	< .001	0.002
AMA 10 µm	p-value	—	0.250	0.019	0.002	0.006	0.800	0.231	0.028
EGF 1 µm+AMA 10 µm	p-value		—	0.623	0.079	0.238	0.942	1.000	0.761
EGF 2 µm+AMA 10 µm	p-value			—	0.732	0.989	0.161	0.657	1.000
EGF 4µm+AMA 10 µm	p-value				—	0.992	0.016	0.086	0.593
EGF 2 µm+ SIL 25 µm+ AMA 10 µm	p-value					—	0.049	0.258	0.952
SIL 12.5µm+ AMA 10 µm	p-value						—	0.926	0.226
SIL 25 µm+AMA 10 µm	p-value							—	0.791

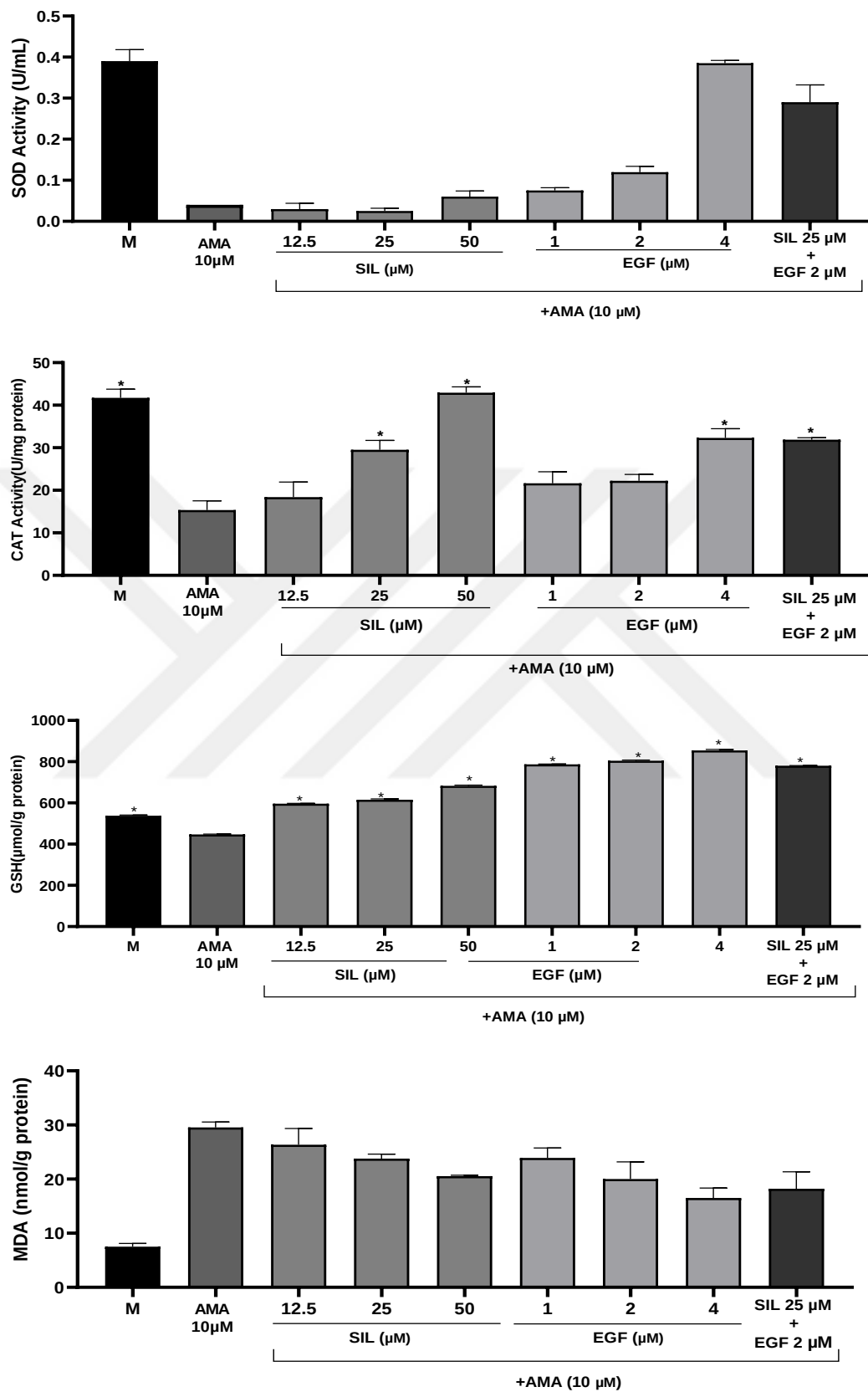


Figure 11. The effect of AMA, EGF and SIL on SOD, CAT, GSH and MDA
M: Control group treated with RPMI; Results were expressed as mean± SD compared to control. The significant differences between groups and AMA were defined with a * $p < 0.05$

Correlation analysis between oxidative stress parameters showed that SOD and CAT have positive strong correlation with MDA levels and it is statistically significant ($p<0.001$). SOD and CAT have a weak positive correlation when compared between themselves ($p=0.024$) (Table 13).

Table 13. The correlation between oxidative stress parameters

	MDA	GSH	SOD
GSH	-0.218 (0.385)		
SOD	-0.827 (<0.001)	0.309 (0.212)	
CAT	-0.729 (<0.001)	0.135 (0.592)	0.527 (0.024)

p values given in brackets. $p<0.05$ is statistically significant.

4.2. *In Vivo* Study Results

4.2.1. Average Weight of Mice

There was statistically difference between the baseline weight averages of the mice in the experiment groups. When the differences between the weights of the mice measured at 48 hours before being sacrificed and their initial weights were also evaluated, and no difference was found in weight difference between the experiment groups (Table 14).

Table 14. Preexperiment postexperiment weights and weight differences of mices

Experiment Groups	Preexperiment Weight (Mean±SD)	Postexperiment Weight (Mean±SD)	Weight difference
Control	37.783±1.68	38.017±1.77	0.233±1.37
AMA	33.850±2.68	31.667±2.20	-2.183±1.33
AMA+EGF750	32.733±2.36	30.150±2.67	-2.583±1.30
AMA+EGF1500	32.433±2.06	32.250±2.71	0.183±1.86
AMA+SIL	35.017±2.21	32.217±2.95	-2.800±3.07
AMA+SIL+EGF750	33.117±1.38	30.400±1.26	-2.717±1.70

4.2.2. Results of Liver Function Tests Values in Experiment Groups

Results of liver function tests AST, ALT, Alkaline Phosphatase (ALP), GGT showed statistically significant differences between groups ($p < 0.001$). Games-Howell post hoc analysis performed to find the cause of difference in which group (Table 15).

AST, ALT, ALP, GGT values; in the EGF 1500 group (2338.0 ± 523.44 U/L, 1105.30 ± 665.48 U/L, 1849.70 ± 533.18 U/L, 159.20 ± 57.41 U/L) $p < 0.05$; Silibinin group (5124.0 ± 1541.56 U/L, 3767.70 ± 957.84 U/L, 5949.30 ± 2778.57 U/L, 300.30 ± 84.34 U/L) $p < 0.05$; Silibinin+EGF 750 group (5217.50 ± 1309.56 U/L, 3715.30 ± 1171.82 U/L, 5500.70 ± 2020.61 U/L, 308.70 ± 79.66 U/L) $p < 0.05$. It was significantly decreased compared to the α -AMA+SF group (7947.0 ± 1335.0 U/L, 6454.0 ± 1604.0 U/L). In the EGF 750 group AST, ALT, ALP, GGT (6590.30 ± 1265.68 U/L, 4841.30 ± 1225.63 U/L, 8799.0 ± 1607.37 U/L, 519.70 ± 112.56 U/L). These measured values did not differ significantly compared to the AMA+SF group $p > 0.05$.

AST and ALT values in EGF 1500 group was significantly decreased compared to the EGF 750 group ($p < 0.05$). Silibinin and Silibinin+EGF750 groups did not differ significantly compared to the EGF 750 group ($p > 0.05$). EGF 1500 group was also significantly decreased compared to Silibinin and Silibinin+EGF750 groups ($p < 0.05$) (Figure 12,13).

ALP values in EGF 1500, Silibinin and Silibinin+EGF750 group was significantly decreased compared to the EGF 750 group ($p < 0.05$). EGF 1500 group was significantly decreased compared to the Silibinin+ EGF 750 group ($p < 0.05$). Also decreased when compared to Silibinin group but there was no significant difference ($p > 0.05$) (Figure 14).

GGT values in EGF 1500 group was significantly decreased compared to the EGF 750 group ($p < 0.05$). Silibinin and Silibinin+EGF750 groups did not differ significantly compared to the EGF 750 group ($p > 0.05$). EGF 1500 group was significantly decreased compared to the Silibinin+ EGF 750 group ($p < 0.05$). Also decreased when compared to Silibinin group but there was no significant difference ($p > 0.05$) (Figure 15).

Table 15. Comparison of laboratory results of mice by experiment groups

	Control	AMA	AMA+EGF750	AMA+EGF1500	AMA+SIL	AMA+SIL+EGF750	p
AST	163.50±46.86	8448.20±1387.59	6590.30±1265.68	2338.0±523.44	5124.0±1541.56	5217.50±1309.56	< .001
ALT	196.0±43.29	7924.70±2437.98	4841.30± 1225.63	1105.30±665.48	3767.70±957.84	3715.30 ±1171.82	< .001
ALP	213.30±85.40	12011.30 ±2451.02	8799.0 ±1607.37	1849.70±533.18	5949.30±2778.57	5500.70±2020.61	< .001
GGT	16.30±7.89	553.20±110.52	519.70±112.56	159.20 ±57.41	300.30±84.34	308.70±79.66	< .001

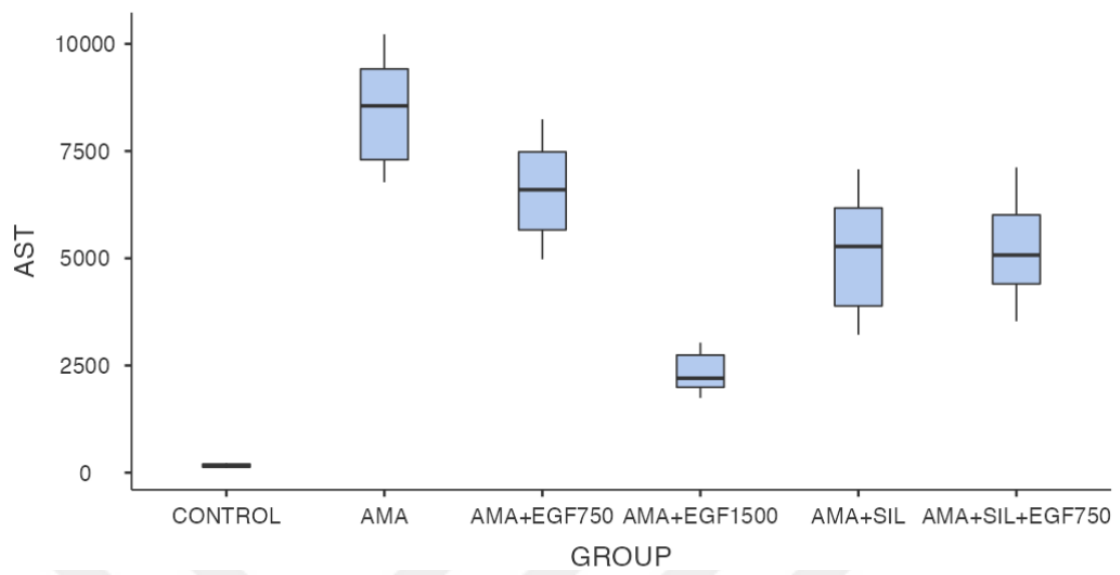


Figure 12. AST (U/L) values in the experiment groups.

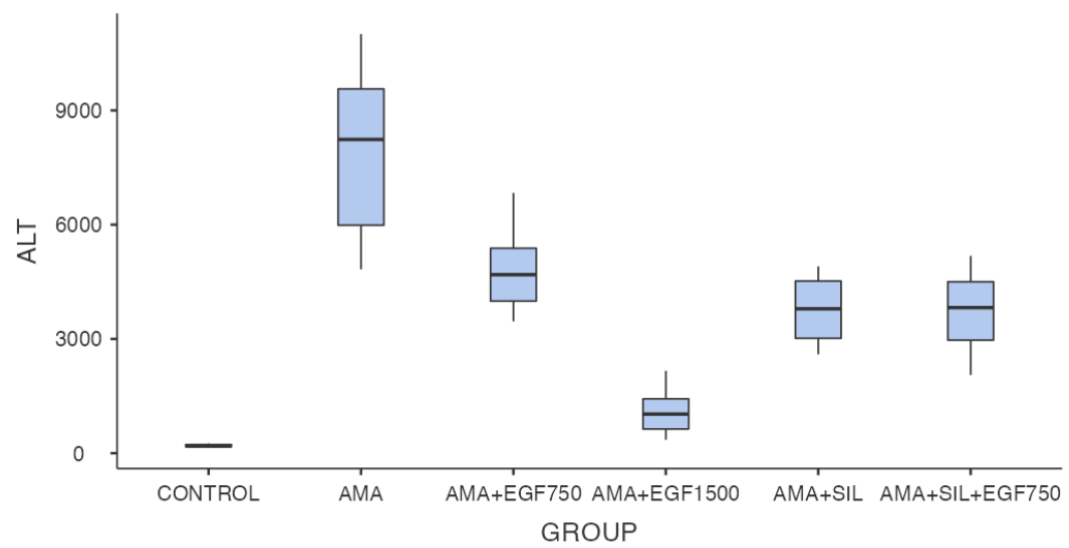


Figure 13. ALT (U/L) values in the experiment groups.

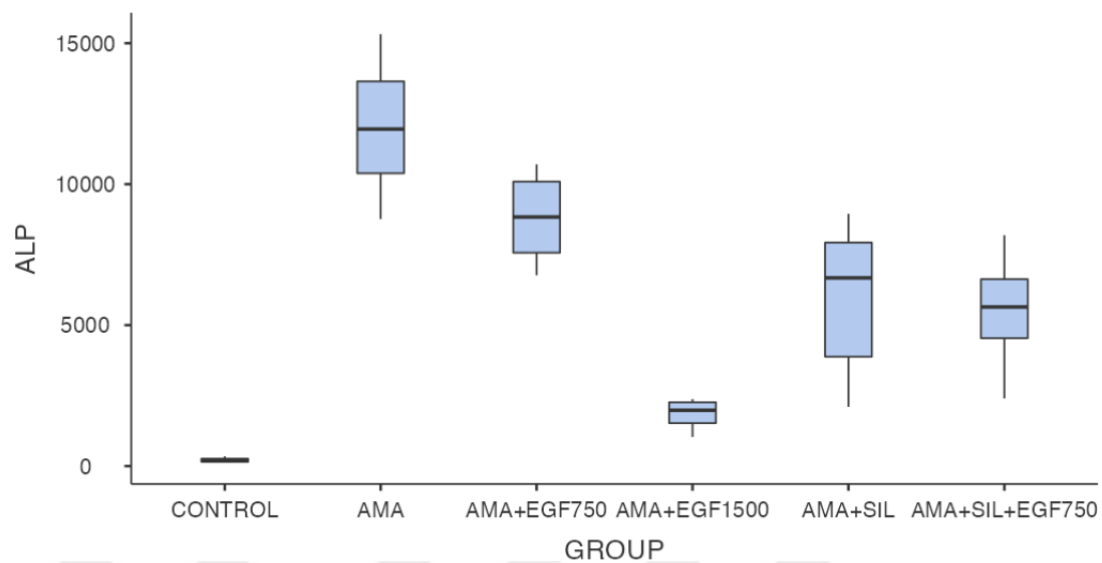


Figure 14. ALP (U/L) values in the experiment groups.

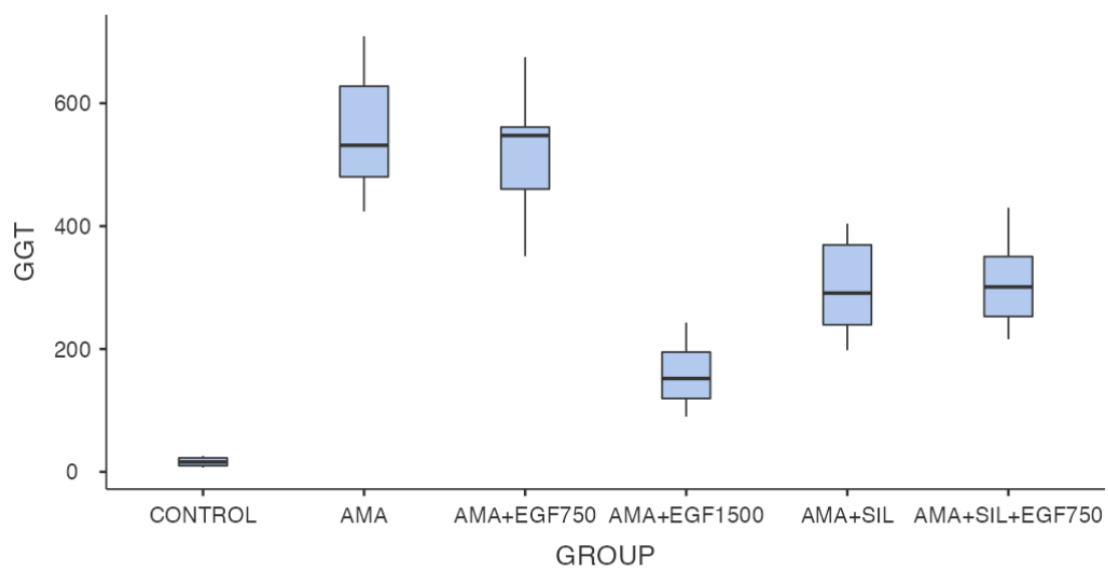


Figure 15. GGT (U/L) values in the experiment groups.

Liver Histomorphological Damage Score and Liver Histomorphology in Experiment Groups

Liver histomorphological damage score showed statistically significant differences between groups. Games-Howell post hoc analysis performed to find the cause of difference in which group.

EGF 1500 (0.667 ± 0.816), decreased significantly compared to AMA group (2.833 ± 0.408) ($p=0.003$). Also, Silibinin+EGF750 (1.167 ± 0.753) combination therapy group decreased LHDS significantly ($p=0.01$). EGF 1500 and combination group has significantly lower LHDS score when compared to EGF750 group ($p<0.05$). EGF 750 (2.667 ± 0.816), Silibinin (1.500 ± 1.643), groups did not differ significantly compared to the AMA+SF group and other groups $p>0.05$ (Table 16) (Figure16). Pothoc analysis (Games Howell) given in Table 17.

Table 16. Comparison of LHDS of mice by experiment groups

	AMA	AMA+ EGF750	AMA+ EGF1500	AMA+ SIL	AMA+SIL+ EGF750	P
LHDS	2.833 ± 0.408	2.667 ± 0.816	0.667 ± 0.816	1.500 ± 1.643	1.167 ± 0.753	<.001

Table 17. Post hoc analysis of LHDS between experimental groups

		AMA	AMA+ EGF750	AMA+ EGF1500	AMA+ SIL	AMA+SIL+ EGF750
Control	p	<.001	0.003	0.447	0.358	0.077
AMA	p	-	0.997	0.005	0.470	0.013
AMA+ EGF750	p		-	0.016	0.644	0.065
AMA+ EGF1500	p			-	0.863	0.870
AMA+ SIL	p				-	0.997

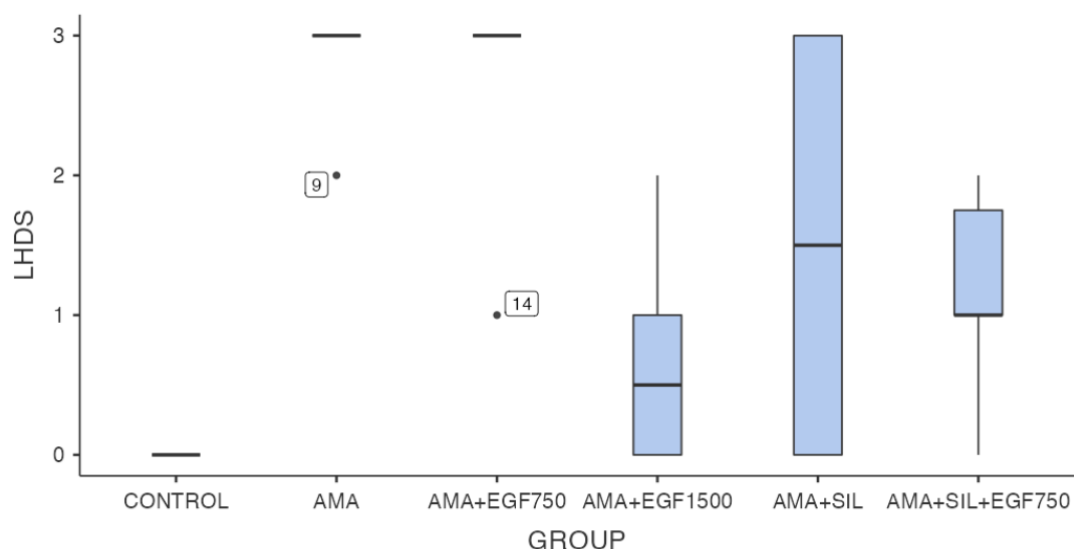


Figure 16. LHDS in the experiment groups

Histomorphological Appearance of Liver Samples in Experiment Groups

Liver cell damage, toxic hepatitis with confluent necrosis were observed in α -AMA+SF group, and diffuse liver cell necrosis and sinusoidal enlargements were observed in liver samples of some mice (Figure 17)

In the EGF 750 group in light microscopic examination, liver cell damage, periportal MNH infiltration, hepatitis characterized by pericentral damage, toxic hepatitis with confluent necrosis were observed. Minimal hepatitis characterized by pericentral damage, usual view, and also just in one case toxic hepatitis with confluent necrosis were observed less in line with the liver histomorphological damage score in EGF 1500 group.

In the histomorphological examination of the Silibinin treatment group, liver cell damage, periportal marked neutrophilic leukocytosis (MNL) cell infiltration, focal necrosis, and sinusoidal enlargements, toxic hepatitis with confluent necrosis were observed.

In the Silibinin+EGF750 treatment group, liver cell damage, periportal MNL cell infiltration, focal necrosis, and sinusoidal enlargements, toxic hepatitis with confluent necrosis were observed (Figure 17).

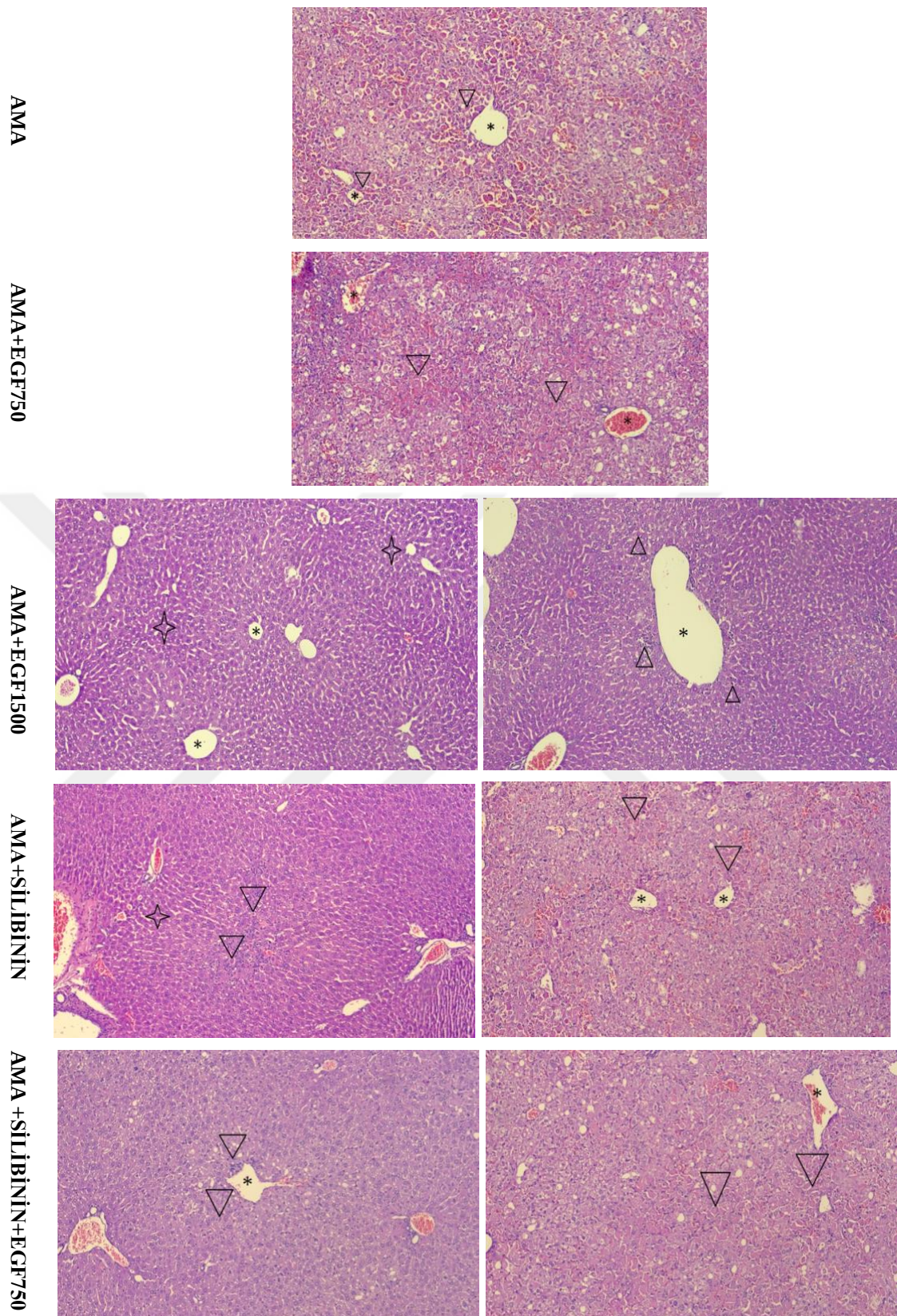


Figure 17. Histomorphological appearance by HE staining of the liver.
 (*) – Central Vein, (▽) – Hepatocyte Injury (◇) - Sinusoidal dilatation

Immunohistochemical Evaluations in Experiment Groups

Perinuclear and cytoplasmic active caspase-3 immunopositivity was increased in α -AMA+SF group (125.83 ± 23.96). It was observed that active caspase-3 immunopositivity was decreased in all treatment groups and showed statistically significant differences between groups. Games-Howell post hoc analysis performed to find the cause of difference in which group (Figure 18).

Caspase-3 Score in EGF 1500 group was significantly decreased compared to the AMA group ($p < 0.05$). EGF750 (80.000 ± 44.944), Silibinin (53.167 ± 62.017) and Silibinin+EGF750 (69.500 ± 73.413) groups did not differ significantly compared to the AMA group ($p > 0.05$). Also, there is no significant difference between all treatment groups ($p > 0.05$) (Table 18) (Figure 19). Post hoc analysis of the difference between groups given in Table 19.

Table 18. Comparison of Caspase 3 Score of mice by experiment groups

	AMA	AMA+EGF750	AMA+EGF1500	AMA+SIL	AMA+SIL+EGF750	p
Caspase-3	125.83 $\pm$ 23.96	80.00 $\pm$ 44.94	15.50 $\pm$ 15.95	53.17 $\pm$ 62.02	43.67 $\pm$ 56.24	< .001

Table 19. Post hoc analysis of Caspase-3 Score between experimental groups.

		AMA	AMA+EGF750	AMA+EGF1500	AMA+SIL	AMA+SIL+EGF750
Control	p	< .001	0.046	0.310	0.407	0.489
AMA	p	-	0.333	< 0.001	0.203	0.095
AMA+EGF750	p		-	0.100	0.948	0.810
AMA+EGF1500	p			-	0.708	0.832
AMA+SIL	p			0	-	1.000

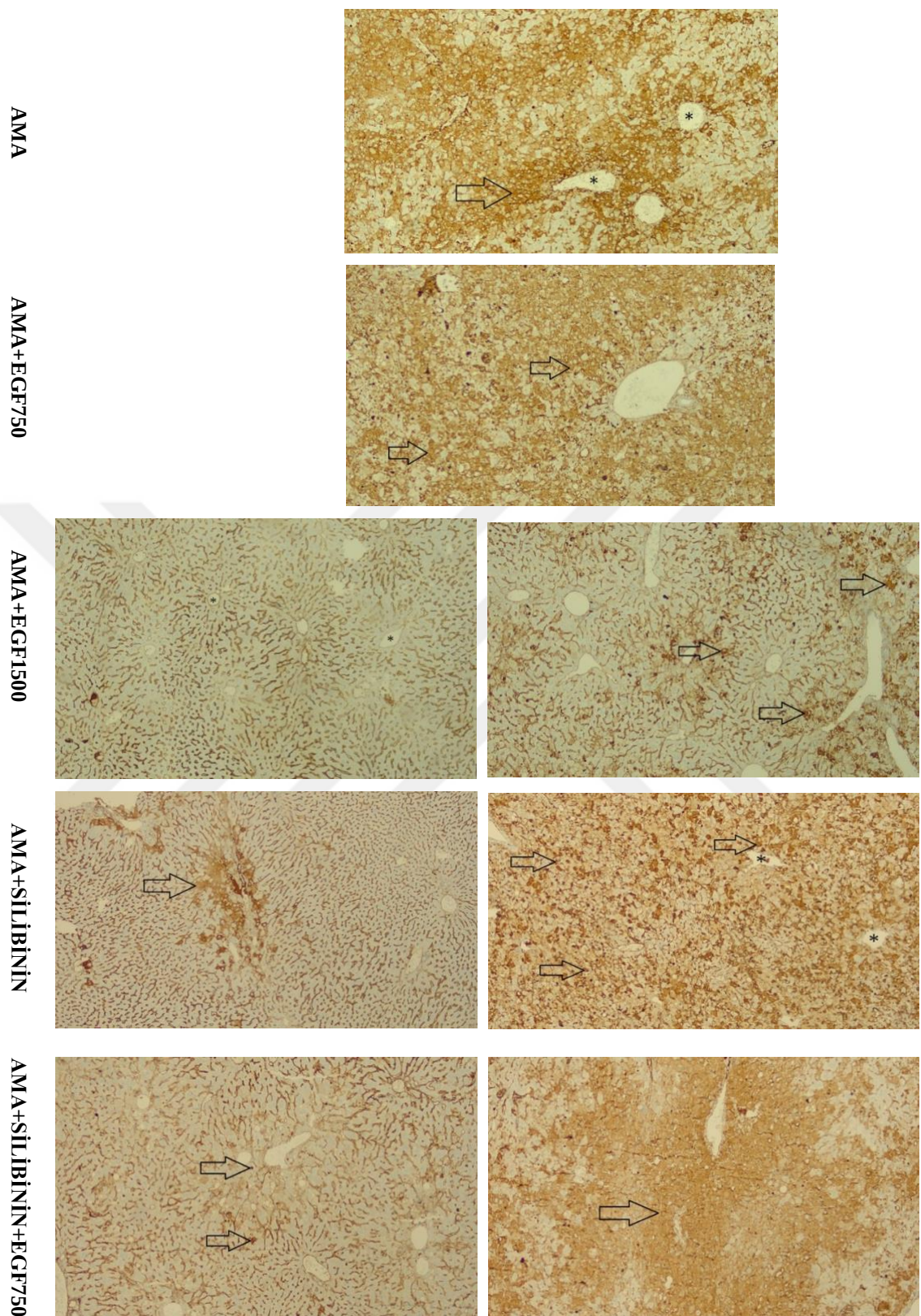


Figure 18. Active caspase-3 immunohistochemical staining of the liver tissue.
 (→) - CASPAS-3 immunoreactivity

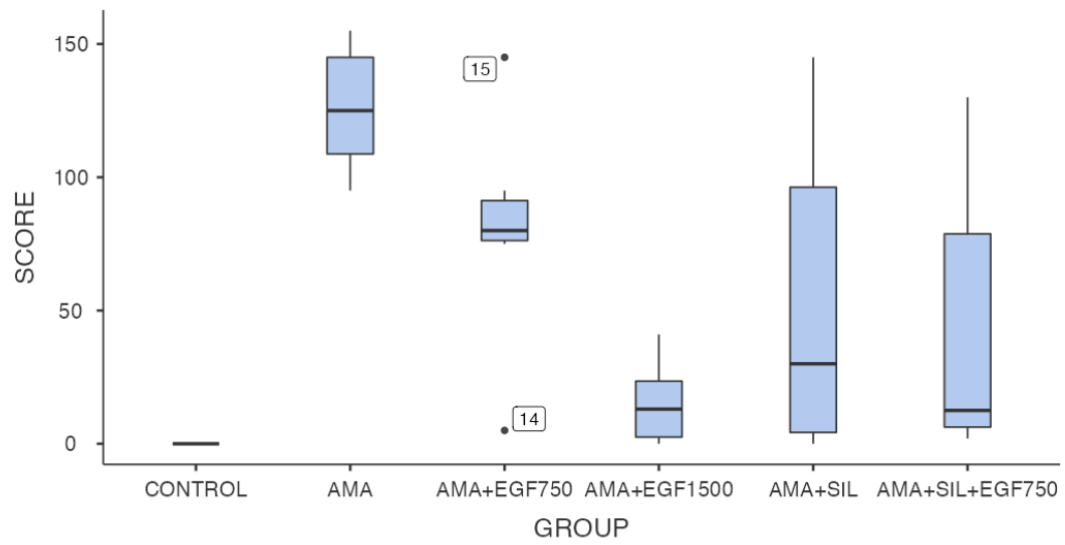


Figure 19. Caspase-3 immunopositivity scores between experiment groups

5. DISCUSSION AND CONCLUSION

Although α -AMA in the content of *Amanita Phalloides* type mushrooms, which causes severe poisoning that ends with death, affects gastrointestinal system epithelial cells, kidney proximal tubule cells, its main target is liver cells, and liver toxicity due to such fungi is responsible for morbidity and mortality. In our study, the effects of EGF on liver toxicity induced by α -AMA in *in vitro* and *in vivo* liver toxicity model were investigated and compared with Silibinin, which is used as an antidote in the clinic (38).

The evaluation of cytotoxicity is essential in understanding the potential harm of substances, particularly in the case of amatoxin poisoning caused by α -AMA. The cytotoxic effects of α -AMA observed in our study align with the known hepatotoxicity of amatoxins. Amatoxins, including α -amanitin, are cyclic peptides found in various poisonous mushroom species, such as *Amanita Phalloides* (death cap). These toxins exert their detrimental effects primarily on liver cells, leading to severe liver damage and potentially fatal outcomes. Research has shown that the toxicity of amatoxins is mediated by their ability to inhibit RNA polymerase II, a key enzyme involved in transcriptional processes. By targeting this enzyme, amatoxins disrupt protein synthesis and cause cellular damage, particularly in hepatocytes (56, 98). In this study, the cytotoxic effects of α -AMA were investigated on HuH-7 cell lines. The cytotoxicity of α -AMA observed in our study further supports its role as a hepatotoxic compound and highlights the importance of caution and prompt medical intervention in cases of amatoxin poisoning. The results revealed that α -AMA exhibited dose-dependent cytotoxicity, with concentrations of 20 μ M and 40 μ M significantly reducing cell viability.

To understand the effectiveness of potential therapeutic interventions, we investigated the cytotoxicity of α -AMA and explored the effects of EGF and SIL in mitigating this cytotoxicity. Consistent with previous research on α -AMA, our results demonstrated a dose-dependent cytotoxic effect on HuH-7 cells (97). However, intriguingly, both EGF and SIL treatments exhibited a protective effect against α -AMA-induced cytotoxicity, as evidenced by increased cell viability. These findings suggest the potential of EGF as therapeutic agents in mitigating the harmful effects of α -AMA.

Previous studies have reported the cytoprotective properties of EGF and SIL in various cellular contexts. EGF, a growth factor involved in cell proliferation and tissue

repair, has been shown to possess anti-apoptotic and regenerative properties in different cell types, including liver cells. EGF can activate survival pathways, such as the PI3K/Akt pathway, and promote cell survival and tissue regeneration (5, 99, 100).

SIL, a flavonolignan derived from milk thistle (*Silybum marianum*), is widely recognized for its hepatoprotective effects and has been extensively studied in the context of cytotoxicity induced by various hepatotoxins. SIL possesses antioxidant, anti-inflammatory, and anti-apoptotic properties, which contribute to its hepatoprotective effects (101, 102). In the case of amatoxin poisoning, SIL has been shown to inhibit the uptake of α -AMA into hepatocytes and mitigate α -AMA-induced liver injury (40, 103). SIL modulates signaling pathways, including the mitogen activated protein kinases (MAPK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathways, to promote cell survival, reduce oxidative stress, and inhibit apoptosis (104). Our results indicated that SIL treatment also resulted in a dose-dependent decrease in cytotoxicity, indicating its potential to protect cell viability.

EGF is a growth factor known for its proliferative and regenerative properties. In the context of cytotoxicity, EGF has been investigated as a potential protective agent against various toxins and hepatotoxic agents. Studies have shown that EGF treatment can enhance cell survival and tissue regeneration in liver injury models. EGF activates survival pathways, such as the PI3K/Akt pathway, which plays a crucial role in promoting cell survival and protecting against cytotoxic insults (5). The protective effects of EGF against liver injury have been demonstrated in models of hepatotoxicity induced by various agents, including acetaminophen and CCl₄ (6, 7). In our study, EGF treatment demonstrated a dose-dependent decrease in cytotoxicity, indicating its potential to protect cell viability.

Both EGF and SIL treatments demonstrated protective effects against α -AMA-induced cytotoxicity in our study. While EGF primarily acts through activation of survival pathways, SIL exerts its effects through multiple mechanisms, including inhibition of α -AMA uptake, antioxidant activity, and anti-apoptotic properties. The combination treatment of EGF and SIL yielded a similar protective profile as high-concentration EGF treatment alone, suggesting that the protective effects of the combination treatment may rely on EGF-mediated pathways. It is important to note that the effectiveness of EGF and

SIL in mitigating cytotoxicity can vary depending on the specific context and experimental models.

MDA is a widely used biomarker of oxidative stress and lipid peroxidation. Elevated MDA levels indicate increased oxidative damage to cellular membranes and are associated with various pathological conditions, including liver injury (105). In our study, EGF treatment demonstrated a dose-dependent decrease in MDA levels, suggesting its potential to reduce oxidative stress caused by α -AMA exposure.

The literature supports the notion that EGF has a protective effect against oxidative stress and lipid peroxidation. Numerous studies have shown that EGF treatment can attenuate oxidative stress-induced damage in different cell types and tissues, including the liver (106, 107). One of the mechanisms by which EGF exerts its antioxidative effects is through the activation of downstream signaling pathways, such as the PI3K/Akt pathway. Activation of the PI3K/Akt pathway by EGF leads to the upregulation of antioxidant enzymes, including SOD and CAT, which play crucial roles in cellular defense against oxidative stress (5). In liver injury models, EGF has shown hepatoprotective effects by reducing MDA levels and enhancing antioxidant capacity. For example, in a study on CCl₄ induced liver injury, EGF treatment significantly decreased MDA levels and increased SOD and CAT activities, leading to reduced oxidative damage and improved liver function (6). Furthermore, in models of liver ischemia-reperfusion injury and acetaminophen-induced hepatotoxicity, EGF administration has been shown to reduce MDA levels and inhibit lipid peroxidation, contributing to the preservation of liver integrity and function (7). In our study, EGF treatment demonstrated a dose-dependent decrease in oxidative stress parameters, suggesting its potential to reduce oxidative stress caused by α -AMA exposure.

The literature provides substantial evidence supporting the antioxidative properties of Silibinin and its ability to reduce MDA levels. Silibinin exerts its antioxidative effects through multiple mechanisms. One of the key mechanisms is its ability to scavenge free radicals and inhibit lipid peroxidation (108). Silibinin can directly neutralize ROS and inhibit the formation of lipid peroxides, thus reducing oxidative stress and subsequent MDA production (109).

In the context of liver injury, Silibinin has demonstrated significant hepatoprotective effects by reducing MDA levels and inhibiting lipid peroxidation. Studies on various liver injury models, including drug-induced hepatotoxicity and liver fibrosis, have consistently shown that Silibinin treatment can attenuate oxidative stress, reduce MDA levels, and improve liver function (110, 111).

For instance, in a study on acetaminophen-induced liver injury, Silibinin administration resulted in a significant reduction in MDA levels, accompanied by enhanced antioxidant enzyme activities and improved liver histology (110). Similarly, in a model of CCl₄ induced liver injury, Silibinin treatment effectively reduced MDA levels and ameliorated oxidative damage in liver tissues (112).

The protective effects of Silibinin against oxidative stress and lipid peroxidation are attributed to its antioxidant, anti-inflammatory, and anti-apoptotic properties. Silibinin can modulate multiple signaling pathways, including the MAPK and PI3K/Akt pathways, which play crucial roles in cellular survival, inflammation regulation, and redox balance (104).

When used in combination, EGF and Silibinin may exert synergistic effects in reducing MDA levels and mitigating oxidative stress. The combined treatment may enhance the activation of intracellular signaling pathways involved in cellular defense mechanisms against oxidative damage. To our knowledge there is no specific studies on the combination of EGF and Silibinin in the context of MDA reduction and effects in other oxidative stress-related conditions.

Studies have shown that Silibinin and EGF treatment can upregulate SOD and CAT activity, thereby enhancing the antioxidant defense system. Silibinin stimulates SOD and CAT expression and activity, leading to increased scavenging of superoxide radicals, which are highly reactive and contribute to oxidative stress (109). EGF treatment can also activate signaling pathways such as the PI3K/Akt pathway which in turn upregulates SOD and CAT expression, leading to increased cellular antioxidant capacity (99).

In our study, a liver toxicity model was created with α -AMA in mice and it was concluded that EGF reduced liver toxicity developed with α -AMA in mice. Although clinically, the liver toxicity developed in α -AMA poisonings and Silibinin used as an

antidote made some improvement in the biochemical parameters, the histomorphological improvement observed in the liver tissue was not reflected.

Our experimental toxicity model was created based on experimental protocols previously applied in the literature. First, it is reported in the literature that half of the mice administered α -AMA cause death. Lethal Dose 50 (LD50) (0.75 mg/kg) was administered intraperitoneally (i.p.) to our experimental groups.

In clinical practice, progressive increase in serum aminotransaminase levels are the most frequently used parameters in diagnosing liver toxicity and showing its severity (89). The most valid methods used in the evaluation of liver necrosis and impaired hepatic function are primarily increases in AST and ALT values, and then ALP and GGT. In our study, we observed an increase in AST, ALT, GGT and ALP values (113-115).

In addition, the fact that we observed a significant increase in liver damage score and liver necrosis histomorphologically apart from elevations of liver function tests are strong evidence that α -AMA causes liver toxicity. Necrosis is an indicator of cell death in living organisms. Toxic substances are also among the factors that cause the development of necrosis. In our study, we have shown that α -AMA causes necrosis in a dose-proportional manner, and therefore creates serious liver toxicity (116).

The target organ of α -AMA is mainly the liver. α -AMA causes toxicity and cell death after entering the cell with carrier proteins called OATP1B3 located in the sinusoidal membrane of liver cells (40). The most basic known mechanism in the development of toxicity is that α -AMA inhibits RNA polymerase II in the liver cell nucleus and prevents mRNA synthesis, preventing DNA transcription and stopping protein synthesis, leading to liver cell necrosis (41).

Other valid mechanisms are that α -AMA induces apoptosis with its synergistic effect on TNF- α , and creates oxidative damage by increasing ROS (54, 56). It has also been reported that α -AMA can induce oxidative damage due to increasing SOD and MDA and decreasing catalase level (56). Therefore, studies to develop an effective antidote in the treatment of α -AMA-induced liver toxicity basically have two purposes:

- First, blocking the entry of α -AMA into liver cells
- The second is to preserve the viability of liver cells by preventing the pathophysiological changes caused by α -AMA.

In our study, we used EGF, which we thought can reverse the pathophysiological changes in the development of α -AMA-induced liver toxicity. EGF, which was shown to be effective in reversing the toxic effects developed in the liver toxicity model with α -AMA. In our *in vivo* experiments, doses of 750 μ g/kg and 1500 μ g/kg used in this study. Since effective results could not be obtained at 750 μ g/kg and at the dose of 1500 μ g/kg doses were applied in the animal experiment.

The higher dose of EGF (1500 μ g/kg), showed better therapeutic effect on liver toxicity was demonstrated by a significant decrease in elevated liver function tests and histomorphological liver damage score compared to the other experimental groups. It has been reported that EGF impairs the cytochrome C-Caspase-3 apoptotic pathway induced by transforming growth factor b in hepatocytes (117) and EGF prevent acute choline deficiency triggered apoptosis in hepatocytes via inhibiting cytochrome C release and restoring mitochondrial membrane potential (118). All these studies suggest that the protection of EGF against these hepatotoxins on hepatocytes may be via modulating apoptotic signal pathway.

In our study, histomorphologically, liver cell damage, periportal MNL infiltration, focal necrosis, diffuse liver cell necrosis and sinusoidal enlargements were observed in liver samples of some mice in α -AMA+SF group. Liver cell damage, periportal MNL infiltration, focal necrosis and sinusoidal enlargements were less in all treatment groups. However statistically significant change was just observed at higher dose EGF group. Therefore, EGF treatment at higher doses reduced the histomorphological damage observed in the liver due to α -AMA.

Necrosis, like apoptosis, is a process that causes cell death in living organisms. In necrosis, which is classical cell death, lysosomal enzymes scattered around as a result of swelling and disintegration of cells due to hypoxia or various toxic factors initiate inflammatory processes. It has been reported that EGF markedly reduced the degree of hepatic injury in a dose-dependent fashion. EGF may therefore prevent CC14-induced hepatic injury by stimulating the production of superoxide dismutase in the hepatocytes.

This idea is supported by the finding that administration of exogenous antioxidants can reduce CCl₄-induced hepatic damage (119). Other mechanisms by which EGF might have exerted its effects include altering blood flow and prostaglandin levels, which have been shown to change in response to administration of EGF during studies on gastric models of injury.

Therefore, EGF has an anti-apoptotic and cell regeneration effect with pathologies developing in liver cells, as in many cells. Histomorphologically, the decrease in MNL infiltration observed in the EGF treatment groups suggests that EGF acts with an anti-apoptotic mechanism in liver toxicity due to α -AMA. In our study, we did not measure serum NF- κ B or Interleukin (IL-1, IL-6) levels, which can confirm inflammation. However, we demonstrated the anti-inflammatory effect of EGF by reducing the infiltration of MNL in liver tissue due to α -AMA.

EGF treatment caused a significant decrease in active Caspase-3 immunopositivity in the EGF 1500 μ g/kg group compared to the α -AMA+SF group. Although caspase-3 immunopositivity was close to the α -AMA+EGF 1500 group in the AMA+EGF 750 μ g/kg and AMA+ Silibinin group, statistical significance was not found in these groups compared to the EGF 1500 μ g/kg treatment group.

Apoptosis is a process that causes the activation of genes and enzymes depending on various physiological and pathological factors. Bcl-2 oncoprotein and p53 protein contribute to the development of apoptosis. In apoptosis, a physiological mechanism that requires energy and causes cell death, certain cells are affected. DNA; DNA fragments to form fragments. Apart from that, specific genes such as Ces-1 and Ces-2 also stimulate apoptosis. Electron microscopy and TUNEL technique are methods used to determine cell apoptosis. Moreover, the changes seen in apoptosis are also determined by the activation of caspases. Caspase-3 is responsible for apoptotic effects and is the most common caspase in cells. In our study, we showed by electron microscopic and immunohistochemical staining that α -AMA causes apoptosis and EGF treatment prevents apoptosis when applied at 1500 levels. Therefore, α -AMA increased apoptosis in liver cells and EGF treatment decreased apoptosis in liver cells due to α -AMA. Antiapoptotic effects of EGF have also been reported in experimental studies. EGF has been shown to prevent clivorine-induced cell apoptosis via mitochondrial-mediated apoptotic signal pathway, which including clivorine decreased antiapoptotic Bcl-xL protein, thus

regulating cytochrome C releasing into cytosol, leading to the activation of caspase-9 and finally apoptosis. EGF also have an effect on clivorine-induced Bcl-xL decrease, cytochrome C release, and caspase-9 activation.

Berlanga et al also reported that EGF prevents apoptosis in liver toxicity developing with CCl₄. Clinical trials of EGF are presently underway for the treatment of ulcerative conditions of the bowel, for conditions such as necrotizing enterocolitis (120). EGF is found in human gastric juice at relatively high concentrations (about 500 ng/l). It probably acts as a 'luminal surveillance' peptide in the gastro- intestinal tract, readily available to stimulate repair at sites of injury when the EGF receptors located on the basolateral membranes of the enterocytes become exposed (121). In contrast, circulating concentrations of endogenous EGF are extremely low, suggesting that we are demonstrating a pharmaco- logical, as opposed to a physiological, action of EGF on its receptor.

It has been reported that exposure to α -AMA for 12 hours in primary dog liver cell cultures causes a decrease in cell viability proportional to the α -AMA concentration and exposure time. In another study, it was shown in human liver cell culture that apoptosis has a role in liver toxicity due to α -AMA and that apoptosis is caspase-3 dependent, which is considered an apoptotic marker and plays an important role in apoptosis (119). Although apoptosis has been reported to be particularly pronounced after 6 hours of exposure, it has been shown that apoptosis and necrosis occur simultaneously in canine liver cell cultures in a manner proportional to the concentration of exposure to α -AMA. Therefore, studies are needed to investigate both the mechanism of apoptosis induced by α -AMA and the mechanism by which EGF reverses this effect. Silibinin is a substance used as an antidote in liver toxicity due to *Amanita Phalloides*. In our study, we observed that Silibinin was effective on histomorphologically developing liver toxicity due to α -AMA, but this effect was not reflected in biochemical values. Histomorphologically, liver cell damage, periportal MNL cell infiltration, focal necrosis, and sinusoidal enlargements were found to be more in the Silibinin group compared to the EGF 1500 μ g/kg treatment groups. Tong et al. also showed that Silibinin, like cimetidine, benzylpenicillin and thioctic acid, is not effective in liver toxicity due to α -AMA in mice (69). However, Vogel et al., in liver toxicity in beagle dogs; They reported that 50 mg/kg Silibinin

administration 5-24 hours after the creation of toxicity improved biochemical enzymes and reduced necrosis in the liver, and there was no death in the Silibinin group (122).

In a retrospective study, in a series of 18 patients, liver toxicity was reported in poisoning with *Amanita Phalloides* type mushrooms, correlated with the length of time between ingestion of the mushroom and administration of Silibinin. However, in this retrospective study, the amount of toxins that patients were exposed to was not standardized, and the results of retrospective studies could not be presented as evidence for evaluating the efficacy of a treatment (123). Another study compared the efficacy of Silibinin alone or in combination with penicillin G in mushroom poisoning of *Amanita Phalloides*. Of the patients included in the study, 118 received Silibinin as monotherapy, while 249 received combined therapy with penicillin G. As a result of the regression analysis, mortality and transplantation rates were found as 5.1% in monotherapy and 8.8% in combined therapy. Although there are publications showing that it is effective, it is not 100% effective (124).

Silibinin is still used as an antidote in poisoning with *Amanita Phalloides* type mushrooms. However, in the toxicity model we created in our study, we did not find Silibinin to be effective on biochemical parameters. There is insufficient evidence for the efficacy of Silibinin in poisonings, whether experimental studies or studies evaluating human data. Therefore, there is a need for new antidotes in poisoning with such fungi.

In recent years, several studies have taken place in the literature to develop antidotal treatment against α -AMA-induced liver toxicity. The first of these is the experimental study of Garcia et al. with polymyxin B (125). According to the results of this study, which included both *in silico* and *in vivo* protocols, it was concluded that polymyxin B was protective against liver failure due to α -AMA-induced liver toxicity and increased the survival rates of mice. However, there are some limitations in the study. Compared to our research; the dose of α -AMA used in the experimental protocols is about one-fourth of the dose we used in our experiment. In our study, we found EGF to be effective in curing α -AMA-induced liver toxicity, although we did not cause liver toxicity with a very high dose of α -AMA compared to the literature. Therefore, we think that even better results can be obtained in case of toxicity with lower doses of α -AMA. Another limitation of this study is that the effects of Polymyxin B were evaluated only 4 hours after α -AMA

administration, and it was not administered later, which could be adapted to the poisoning observed in humans.

Another study on the investigation of new treatments in liver toxicity due to α -AMA is the study of Kaya et al. (120). In this study, the ameliorating effect of erdosteine on liver toxicity was investigated in an *in vivo* liver toxicity model induced by α -AMA in mice. In this study, the dose of α -AMA applied in the experimental protocols was one-third of the dose erdosteine were used, and it was started to be applied approximately 3 hours after exposure to toxin. Therefore, in this study, the late-term ameliorating effect of erdosteine in liver toxicity due to α -AMA was not examined. However, EGF provided statistically significant improvement in both aminotransferases and histomorphological damage scores even when administered 6 hours after α -AMA administration in our experimental protocol.

We have some limitations in our experimental study, especially clinical adaptation. The main limitations of our study;

- The maximum amount of blood that can be taken from mice is approximately 1 mL (94). Since we created a liver toxicity model in our study, we predicted that the hemodynamics of the mice would be impaired and blood collection would be more difficult. Therefore, more biochemical parameters and coagulation tests could not be evaluated.
- According to the 3R rule (95), we were not able to evaluate the combination of high-dose EGF and Silibinin because we limited the number of mice used in the experiments to the number of animals.
- In our study, although Silibinin EGF combination gave better results than only Silibinin, it could not reach statistical significance.

In conclusion, our *in vitro* study demonstrates the protective effects of EGF and SIL against AMA-induced cytotoxicity in HuH-7 hepatocellular carcinoma cells. These findings highlight the potential of EGF and SIL as therapeutic agents in mitigating the harmful effects of AMA and provide a basis for further research in developing treatment strategies for amatoxin poisoning. In *in vivo* study; we reported that high dose EGF was found the most effective antidote treatment in hepatotoxicity caused by α -AMA

poisoning. Also, it was observed that Silibinin and EGF combination is effective in the treatment as Silibinin. The lowest Liver Function Test results, Caspase-3 score and LHDS due to α -AMA poisoning was observed in high dose EGF group. Therefore, it can be suggested that EGF in high doses can reduce treat the hepatotoxicity due to α -AMA poisoning. Meanwhile, Low dose EGF was found to be insufficient in the treatment. Silibinin only and Silibinin and EGF combination showed promising result but they did not show statistically significance.

On the other hand, Silibinin only and Silibinin and EGF combination showed significantly lower AST, ALT, ALP, GGT when compared to α -AMA. Finally, high dose EGF significantly treats α -AMA poisoning when compared to Silibinin only and Silibinin and EGF combination. Low dose EGF cannot treat the α -AMA poisoning on its own. Silibinin only and Silibinin and EGF combination have improved the liver function tests but cannot effectively treat the liver tissue. Therefore, it can be concluded that the use of EGF in high doses can be used to treat hepatotoxicity due to α -AMA poisoning.

In order to make a detailed comment, while our findings provide valuable insights into the potential therapeutic utility of EGF and SIL in amatoxin poisoning, it is important to note that further studies are warranted. Additional research is needed to elucidate the underlying molecular mechanisms of EGF and that activating survival pathways, such as the PI3K/Akt pathway, exert their protective effects against AMA-induced cytotoxicity. further studies may be repeated by some changes in experimental design such as considering Silibinin with high dose EGF combination compared to high dose EGF only for more promising results. Additionally, optimal dosages and treatment regimens may need to be further investigated and validated in preclinical and clinical studies.

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7. APPENDICES

7.1. Ethical Approval



T.C.
YEDİTEPE ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu (HADYEK)

ETİK KURUL KARARI

Protokol No	Toplantı Tarihi	Toplantı Sayısı	Karar No	Proje Yürütücüsü
2022-48	11.10.2022	2022/10	2022/10-7	Prof. Dr. Hande Sipahi
'Amatoksine Bağlı Hepatotoksisite Tedavisinde Epidermal Büyüme Faktörü Kullanımının İncelenmesi' isimli proje oy birliğiyle etik açıdan koşullu olarak uygun görülmüştür.				
Hayvan Türü / Irkı		Toplam Hayvan Sayısı		Hayvanın Cinsiyeti
Fare / BALBc		36		Erkek

Görevi	Adı Soyadı	Katılım Durumu
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA	
Üye	Dr. Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Prof. Dr. Rukset ATTAR	KATILMADI
Üye	Prof. Dr. Gamze TORUN KÖSE	
Üye	Prof. Dr. Özlem MALKONDU	
Üye	Doç. Dr. Aylin YABA UÇAR	
Üye	Doç. Dr. Burcu GEMİCİ BAŞOL	
Üye	Atakan Mücahit YAVUZ	
Üye	Ahmet ŞENKARDEŞLER	

8. CURRICULUM VITAE

Personal Information

Name	Sinan	Surname	Karacabey
Place of Birth	Bursa	Date of Birth	20.12.1984
Nationality	TC	TC Identity No	71077074294
E-mail	karacabeysinan@gmail.com	Phone Number	+905069138766

Education

Level	Proficiency	Institutions	Date of graduation
PhD	Pharmaceutical Toxicology	Yeditepe University	
Proficiency	Emergency Medicine	Kartal Dr Lutfi Kırdar EAH	2013
Bachelor's Degree		Uludağ Üniversitesi Tıp Fakültesi	2008
High School	-	Bursa Milli Piyango Anadolu Lisesi	2002

Language	Exam and Grade
English	YOKDİL 2017 – 87.5

Work Experience

Duty	Institution	Dates
Associate Professor	Marmara University School of Medicine Department of Emergency Medicine	2019-present
Assistant Professor	Marmara University School of Medicine Department of Emergency Medicine	2016-2019
Assistant Professor	Bozok University School of Medicine Department of Emergency Medicine	2015-2016
Consultant	Bozok University School of Medicine Department of Emergency Medicine	2014-2015

Computer Skills

Program	Ability to use
Microsoft Word, Microsoft Excel, Microsoft powerpoint	Advanced
SPSS, Jamovi	Advanced

1. KARACABEY SİNAN, SANRI ERKMAN (2022). Optic Nerve Sheath Diameter Affected by Cervical Collar Placement in Minor Head Trauma Patients. Iranian Red Crescent Medical Journal, Doi: 10.32592/ircmj.2022.24.10.1530
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4. KARACABEY SİNAN, Sanrı Erkman (2020). Use of Ultrasound for Diagnosis of Clavicle Fractures in the Emergency Department. Signa Vitae
5. Sanrı Erkman, KARACABEY SİNAN (2019). The Impact of Backboard Placement on Chest Compression Quality: A Mannequin Study. Prehospital and Disaster Medicine, 34(02), 182-187., Doi: 10.1017/S1049023X19000153
6. Sanrı Erkman, KARACABEY SİNAN (2019). The Impact of Head of Bed Elevation on Optic Nerve Sheath Diameter in Cervical Collar Applied Healthy Volunteers. The Journal of Emergency Medicine, 56(4), 371-377., Doi: 10.1016/j.jemermed.2018.12.043
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8. Sanrı Erkman, KARACABEY SİNAN, Eroğlu Serkan Emre, AKOĞLU HALDUN, DENİZBAŞI ALTINOK ARZU (2018). The additional impact of simulation based medical training to traditional medical training alone in advanced cardiac life support: a scenario based evaluation. Signa Vitae
9. KARACABEY SİNAN, Sanrı Erkman, YALÇINLI SERCAN, AKOĞLU HALDUN (2018). Which is more effective for the treatment of Acute Migraine Attack: Dexametoprolfen, Ibuprofen or Metoclopramide. Pakistan Journal of Medical Sciences, 34(2), 418-423., Doi: <https://doi.org/10.12669/pjms.342.13815>
10. KARACABEY SİNAN, Sanrı Erkman, Eroğlu Serkan Emre, DENİZBAŞI ALTINOK ARZU (2018). Evaluation of Simulation Training in Paramedic Students for Advanced Airway Training. Scholar Journal of Applied Medical Sciences, 6(4), 1572-1574., Doi: 10.21276/sjams.2018.6.4.39
11. 16. Sanrı Erkman, Akoglu Ebru Unal, KARACABEY SİNAN, VERİMLİ URAL, AKOĞLU HALDUN, ŞEHİRLİ ÜMİT SÜLEYMAN, DENİZBAŞI ALTINOK ARZU (2018). Diagnostic utilities of tracheal ultrasound and USBendoscope for the confirmation of endotracheal tube placement: A cadaver study. AMERICAN JOURNAL OF EMERGENCY MEDICINE, Doi: 10.1016/j.ajem.2018.02.015
12. KARACABEY SİNAN, sanrı erkman, guneyssel ozlem (2016). A Non invasive Method for Assessment of Intravascular Fluid Status: Inferior Vena Cava Diameters and Collapsibility Index. Pakistan Journal of Medical Sciences, 32(4), 836-840., Doi: 10.12669/pjms.324.10290
13. KARACABEY SİNAN, sanrı erkman, gencer emin gokhan, guneyssel ozlem (2016). Tracheal ultrasonography and ultrasonographic lung sliding for confirming endotracheal tube placement Speed and Reliability. The American Journal of Emergency Medicine, 34(6), 953-956., Doi: 10.1016/j.ajem.2016.01.027.
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Other scientific research

1. BAYRAM NURSELİ, AKOĞLU HALDUN, SANRI ERKMAN, KARACABEY SİNAN, EFEÖĞLU MELİS, ONUR ÖZGE ECMEL, DENİZBAŞI ALTINOK ARZU (2021). Diagnostic Accuracy of the Electrocardiography Criteria for Left Ventricular Hypertrophy (Cornell Voltage Criteria, Sokolow-Lyon Index, RomhiltEstes, and Peguero-Lo Presti Criteria) Compared to Transthoracic Echocardiography. *Cureus*, 13(3), Doi: 10.7759/cureus.13883
2. efeoğlu melis, KARACABEY SİNAN, Sanrı Erkman, ömercikoğlu serhad, Ünal Emir, ONUR ÖZGE ECMEL, AKOĞLU HALDUN, DENİZBAŞI ALTINOK ARZU (2021). Variables Affecting Mortality Among COVID-19 Patients With Lung Involvement Admitted to the Emergency Department. *Cureus Journal of Medical Science*, 13(1), 1-8., Doi: 10.7759/cureus.12559
3. Gul Fethi,Sayan Ismet,ARIKAN HÜSEYİN,KARACABEY SİNAN,ARSLANTAŞ MUSTAFA KEMAL,CİNEL İSMAİL HAKKI (2019). The Role of Transocular Ultrasound in The Assessment of Neurotoxicity Related Encephalopathy in Mushroom Poisoning. *Turkish Journal of Anaesthesiology and Reanimation*, Doi: 10.5152/TJAR.2019.37891
4. KARACABEY SİNAN,Sarı Erkman,METİN BAYRAM,ERKOÇ MUSTAFA FATİH,yıldırım şener,İNTEPE YAVUZ SELİM,YALÇINLI SERCAN (2018). Use of ultrasonography for differentiation between bullae and pneumothorax. *Emergency Radiology*, Doi: 10.1007/s10140-018-1640-9 .
5. Sanrı Erkman,KARACABEY SİNAN,DENİZBAŞI ALTINOK ARZU (2018). Red Cell Distribution Width in Predicting 30-Day Mortality in Acute Pulmonary Embolism Patients. *Scholar Journal of Applied Medical Sciences*, 6(6), 2000-2005., Doi: 10.21276/sjams.2018.6.5.27
6. Sanrı Erkman,KARACABEY SİNAN,Akogu Haldun,Kaya Bora,Guneysel Ozlem (2017). Comparison of ketamine/propofol (ketofol) and etomidate/fentanyl (etofen) combinations for procedural sedation and analgesia in the emergency department: An observational study. *Turkish Journal of Emergency Medicine*, 17(3), 89-94., Doi: 10.1016/j.tjem.2017.01.003
7. SERİN HALİLİBRAHİM,DALTABAN İSKENDER SAMET,KARACABEY SİNAN,okur aylin,GÖÇMEN AYŞE YEŞİM,AK HAKAN (2016). Clinical Correlation between Back Pain and Schmorl's Node – Modic's Changes. *Scholars Journal of Applied Medical Sciences (SJAMS)*, 4(11c), 4052-4057
8. Sanrı Erkman,KARACABEY SİNAN,çetin murat,ONUR ÖZGE ECMEL,DENİZBAŞI ALTINOK ARZU (2018). The Analysis of Articles in A Medical Blog: A Retrospective Study. *Anatolian Journal of Emergency Medicine*, 1(1), 14-16.
9. Sanrı Erkman,KARACABEY SİNAN,AKOĞLU HALDUN (2018). Do Hemogram Parameters Correlate With The Level and Location of The Obstruction in Acute Mesenteric Ischemia Patients?. *Acibadem Universitesi Saglik Bilimleri Dergisi*, 0-0., Doi: 10.31067/0.2018.76
10. Taşyürek Tanju,KARACABEY SİNAN,Sarı Erkman,kabaroğlu kerem ali,AKOĞLU HALDUN,ONUR ÖZGE ECMEL,DENİZBAŞI ALTINOK ARZU (2018). Koroner Anjiyografi Hastalarındaki Damar Patolojilerinin Başvuru Anındaki VitalBulgularının, Hikayesinin, Ek Hastalıklarının, Kullandığı İlaçların ve EKGBulgularının Oluşturduğu Risk Oranlarının Tespiti. *Anatolian Journal of Emergency Medicine*
11. KARACABEY SİNAN,Sarı Erkman (2018). Pankreatitte Eritrosit Dağılım Genişliği (RDW)'nin Mortaliteyi Öngörmedeki Değerliliği. *Bozok Tıp Dergisi*, 8(1), 109-113.

International Conference Proceedings

1. KARAVİN EREN ONUR,altunbaş erhan,Sarı Erkman,ÖZPOLAT ÇİĞDEM,KARACABEY SİNAN,demir hasan,AKOĞLU HALDUN,DENİZBAŞI ALTINOK ARZU,ONUR ÖZGE ECMEL (2019). Wolff-ParkinsonWhite After Adenosine for Supraventricular Tachycardia. 2nd Southeast European Congress of Emergency Medicine and Disaster Medicine (Özet Bildiri/Poster)
2. GÖRMAN ÖZGEÇAN,ÖZPOLAT ÇİĞDEM,YILMAZ MUHAMMET,YILDIRIM YAĞMUR,KARACABEY SİNAN,adaç musa,ONUR ÖZGE ECMEL,DENİZBAŞI ALTINOK ARZU (2019). Subarachnoid Hemorrhage Precipitated with Ear Piercing. 2nd Southeast European Congress of Emergency Medicine and Disaster Medicine (Özet Bildiri/Poster)
3. AKKAYA AYSEL İREM,Sarı Erkman,KARACABEY SİNAN,ömercikoğlu serhad,efeoğlu melis,demir hasan,altunbaş erhan,ÖZPOLAT ÇİĞDEM (2018). Pneumocystis Jirovecii Pneumonia in A Refugee. 6th Eurasian Congress on Emergency Medicine 14. Türkiye Acil Tıp Kongresi. (Özet Bildiri/Poster)

4. altunbaş erhan,adañç musa,demir hasan,KARACABEY SİNAN,Sarı Erkman,ÖZPOLAT ÇİĞDEM,AKOĞLU HALDUN,DENİZBAŞI ALTINOK ARZU (2018). Giant Cell Arteritis with Vision Loss due to Delayed Submission. 6th EURASIAN CONGRESS ON EMERGENCY MEDICINE 14th TURKISH EMERGENCY MEDICINE CONGRESS (Özet Bildiri/Poster)
5. emir ünal,SESİĞÜR SU İZEL,KARACABEY SİNAN,Sarı Erkman,AKOĞLU HALDUN,ONUR ÖZGE ECEMEL,DENİZBAŞI ALTINOK ARZU (2018). Drug-induced Acute Pancreatitis Related to Amoxicillin/Clavulanic Acid: Case Report. 6th EURASIAN CONGRESS ON EMERGENCY MEDICINE 14th TURKISH EMERGENCY MEDICINE CONGRESS (Özet Bildiri/Poster)
6. turan yusuf,emir ünal,ÖZTÜRK YASEMİN,KARACABEY SİNAN,Sarı Erkman,AKOĞLU HALDUN,ONUR ÖZGE ECEMEL,DENİZBAŞI ALTINOK ARZU (2018). Foreign Body on the Chest Wall: Case report.. 6th EURASIAN CONGRESS ON EMERGENCY MEDICINE 14th TURKISH EMERGENCY MEDICINE CONGRESS (Özet Bildiri/Poster).
7. emir ünal,atalay halil ibrahim,Sarı Erkman,KARACABEY SİNAN,ömercikoğlu serhad,ÖZPOLAT ÇİĞDEM,ONUR ÖZGE ECEMEL (2018). An Unexpected Adverse Effect of Nilotinib: Rhabdomyolysis.. 6th Eurasian Congress on Emergency Medicine 14. Türkiye Acil Tıp Kongresi. (Özet Bildiri/Poster)
8. BAYAZITLI YİĞİT EŞREF,ömercikoğlu serhad,KARACABEY SİNAN,Sarı Erkman,altunbaş erhan,ÖZPOLAT ÇİĞDEM,AKOĞLU HALDUN,ONUR ÖZGE ECEMEL,DENİZBAŞI ALTINOK ARZU (2018). A Rare Diagnose: Spontaneous Adrenal Gland Hemorrhage. 6th Eurasian Congress on Emergency Medicine 14. Türkiye Acil Tıp Kongresi (Özet Bildiri/Poster)
9. ÖZPOLAT ÇİĞDEM,demir hasan,ömercikoğlu serhad,Sarı Erkman,kabaroğlu kerem ali,KARACABEY SİNAN,ONUR ÖZGE ECEMEL (2018). Amanitin Intoxication: Lucky Woman Alive.. 6th EURASIAN CONGRESS ON EMERGENCY MEDICINE 14th TURKISH EMERGENCY MEDICINE CONGRESS (Özet Bildiri/Poster)
10. KARACABEY SİNAN,Sarı Erkman (2018). Does Cervical Collar Increases The Optic Nerve Sheath Diameter in Minor Head Trauma Patients?. 6th EURASIAN CONGRESS ON EMERGENCY MEDICINE 14th TURKISH EMERGENCY MEDICINE CONGRESS (Özet Bildiri/Sözlü Sunum)
11. GÖRMAN ÖZGEÇAN,ömercikoğlu serhad,altunbaş erhan,KARACABEY SİNAN,ÖZPOLAT ÇİĞDEM,Sarı Erkman,AKOĞLU HALDUN,ONUR ÖZGE ECEMEL,DENİZBAŞI ALTINOK ARZU (2018). Swollen Neck: A Superior Vena Cava Syndrome. 6th Eurasian Congress on Emergency Medicine 14. Türkiye Acil Tıp Kongresi (Özet Bildiri/Poster)
12. turan yusuf,emir ünal,KARACABEY SİNAN,Sarı Erkman,ömercikoğlu serhad,ÖZPOLAT ÇİĞDEM,DENİZBAŞI ALTINOK ARZU (2018). Appendicitis Developed After Abdominal Blunt Trauma. 6th EURASIAN CONGRESS ON EMERGENCY MEDICINE 14th TURKISH EMERGENCY MEDICINE CONGRESS (Özet Bildiri/Poster)
13. AKKAYA AYSEL İREM,efeoğlu melis,Sarı Erkman,KARACABEY SİNAN,ömercikoğlu serhad,ÖZPOLAT ÇİĞDEM,DENİZBAŞI ALTINOK ARZU (2018). Pneumomediastinum Together with Pneumothorax Following Penetrating Neck Injury.. 6th EURASIAN CONGRESS ON EMERGENCY MEDICINE 14th TURKISH EMERGENCY MEDICINE CONGRESS (Özet Bildiri/Poster)
14. GÖRMAN ÖZGEÇAN,KARAVİN EREN ONUR,KARACABEY SİNAN,CEYLAN EZGİ,SESİĞÜR SU İZEL,AKOĞLU HALDUN,ONUR ÖZGE ECEMEL,DENİZBAŞI ALTINOK ARZU (2018). A Rare Reason of Angina Pectoris: Takotsubo Cardiomyopathy. 6th Eurasian Congress on Emergency Medicine 14. Türkiye Acil Tıp Kongresi (Özet Bildiri/Poster)
15. YILDIRIM YAĞMUR,yelmi kadir,emir ünal,KARACABEY SİNAN,Sarı Erkman,ömercikoğlu serhad,ÖZPOLAT ÇİĞDEM,AKOĞLU HALDUN (2018). Facial Trauma After Fall: Le Fort 3. 6th EURASIAN CONGRESS ON EMERGENCY MEDICINE 14th TURKISH EMERGENCY MEDICINE CONGRESS (Özet Bildiri/Poster)
16. çınar ökkeş yılmaz,ÖZPOLAT ÇİĞDEM,efeoğlu melis,Sarı Erkman,KARACABEY SİNAN,ömercikoğlu serhad,AKOĞLU HALDUN,ONUR ÖZGE ECEMEL (2018). Retrospective Analysis of Intoxication Patients. 6th EURASIAN CONGRESS ON EMERGENCY MEDICINE 14th TURKISH EMERGENCY MEDICINE CONGRESS (Özet Bildiri/Sözlü Sunum)
17. emir ünal,Turan Yusuf,Sahbat Yavuz,Sarı Erkman,KARACABEY SİNAN,AKOĞLU HALDUN,ONUR ÖZGE ECEMEL,DENİZBAŞI ALTINOK ARZU (2018). Case Report of a Compartment Syndrome Related to Low Voltage Electrical Injury. 6th EURASIAN CONGRESS ON EMERGENCY MEDICINE 14th TURKISH EMERGENCY MEDICINE CONGRESS (Özet Bildiri/Poster)
18. KARACABEY SİNAN,Sarı Erkman (2018). Spontaneous Subarachnoid Hemorrhage. 6th EURASIAN CONGRESS ON EMERGENCY MEDICINE 14th TURKISH EMERGENCY MEDICINE CONGRESS (Özet Bildiri/Sözlü Sunum)
19. demir hasan,ÇELİK İPEK,altunbaş erhan,Sarı Erkman,KARACABEY SİNAN,ömercikoğlu serhad,ÖZPOLAT ÇİĞDEM,ONUR ÖZGE ECEMEL (2018). Purple Urine Bag Syndrome. 6th Eurasian Congress on Emergency Medicine 14. Türkiye Acil Tıp Kongresi (Özet Bildiri/Poster)
20. ÖZPOLAT ÇİĞDEM,ALTUN MUSTAFA,KARACABEY SİNAN,Sarı Erkman,ömercikoğlu serhad,AKOĞLU HALDUN,ONUR ÖZGE ECEMEL,DENİZBAŞI ALTINOK ARZU (2018). Metformin Intoxication: Case Report.. 6th EURASIAN CONGRESS ON EMERGENCY MEDICINE 14th TURKISH EMERGENCY MEDICINE CONGRESS (Özet Bildiri/Poster)

21. Sanrı Erkman,KARACABEY SİNAN (2018). The Impact Of Backboard Placement On Chest Compression Quality: A Mannequin Study.. 6th EURASIAN CONGRESS ON EMERGENCY MEDICINE 14th TURKISH EMERGENCY MEDICINE CONGRESS (Özet Bildiri/Sözlü Sunum)
22. KARACABEY SİNAN,Sarı Erkman (2018). Ultrasonography in the Diagnosis of Clavicle Fracture. EUSEM 2018, 12th European Emergency Medicine Congress (Özet Bildiri/Sözlü Sunum)
23. YILMAZ MUHAMMET,özcan burak,ömercikoğlu serhad,KARACABEY SİNAN,AKOĞLU HALDUN,ONUR ÖZGE ECMEL,DENİZBAŞI ALTINOK ARZU (2017). Secondary Syphilis: A Case of Hyperemic Conjunctiva with Oral and Limb Lesions. 9th Asian Conference on Emergency Medicine 13th Turkish Emergency Medicine Congress (Özet Bildiri/Poster)
24. emir ünal,çınar ökkeş yılmaz,SESİĞÜR SU İZEL,KARACABEY SİNAN,AKOĞLU HALDUN,ONUR ÖZGE ECMEL,DENİZBAŞI ALTINOK ARZU (2017). A Rare Finding in Neurobrucellosis: Brain Abscess. 9th Asian Conference on Emergency Medicine 13th Turkish Emergency Medicine Congress (Özet Bildiri/Poster)
25. DEMİR OĞUZHAN,akbaş fatih mehmet,ömercikoğlu serhad,KARACABEY SİNAN,ONUR ÖZGE ECMEL,AKOĞLU HALDUN,DENİZBAŞI ALTINOK ARZU (2017). What is the Reason for Syncope?. 9th Asian Conference on Emergency Medicine 13th Turkish Emergency Medicine Congress (Özet Bildiri/Poster)
26. çınar ökkeş yılmaz,emir ünal,ÖZPOLAT ÇİĞDEM,ömercikoğlu serhad,kabaroğlu kerem ali,efeoğlu melis,KARACABEY SİNAN,DENİZBAŞI ALTINOK ARZU (2017). A Rare Cause for Pain in the Lateral Neck: External Jugular Vein Thrombosis. 9th Asian Conference on Emergency Medicine 13th Turkish Emergency Medicine Congress (Özet Bildiri/Poster)
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