T.C.
YEDITEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHARMACEUTICAL TOXICOLOGY

# INVESTIGATION OF REPEATED DOSE TOXICITY OF RAW AND PROCESSED *Lupinus albus* L.

DOCTOR OF PHILOSOPHY THESIS

MERT GÜNAY, B. PHARM.

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This study has approved as a Doctorate Thesis in regard to content and quality by the Jury.

| | Title, Name-Surname (Institution) |
|---|---|
| Chair of the Jury: | Prof. Dr. Ahmet AYDIN, Yeditepe University |
| Supervisor: | Prof. Dr. Ahmet AYDIN, Yeditepe University |
| Member/Examiner: | Prof. Dr. Turgay ÇELİK, Yeditepe University |
| Member/Examiner: | Prof. Dr. Gül ÖZHAN, İstanbul University |
| Member/Examiner: | Prof. Dr. Sibel ÖZDEN, İstanbul University |
| Member/Examiner: | Prof. Dr. Muhammed HAMİTOĞLU, Yeditepe University |

APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated ..... and numbered .....

Prof. Dr. Bayram YILMAZ

Director of Institute of Health Sciences

## DECLARATION

I hereby affirm that this thesis is the result of my original effort, and I believe and acknowledge that it does not include any content that has been previously published or authored by someone else. Additionally, I confirm that no portion of this work has been submitted for the purpose of obtaining any other academic degree, except where appropriate references have been provided within the text.

30.10.2023

Mert GÜNAY

## BEYAN

Bu çalışma kendi gayretimle ortaya koyduğum bir tezdir. Planlamasından yazımına kadar olan süreçte herhangi bir etik dışı davranışta bulunmadığımı vurgularım. Tezde sunulan tüm bilgilerin akademik ve etik standartlara uygun şekilde elde edildiğini belirtirim. Tez çalışması sırasında dışarıdan alınmış herhangi bir bilgi veya yorumun kaynaklarla desteklendiğini ve bu kaynakların ayrıntılı bir şekilde kaynakça bölümünde yer aldığını beyan ederim. Ayrıca, tez hazırlama ve yazma sürecinde patent veya telif haklarını ihlal edecek şekilde herhangi bir davranışta bulunmadığımı ifade ederim.

30.10.2023

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## LIST OF SYMBOLS AND ABBREVIATIONS

α-Tocopherol: Alpha-Tocopherol

8OHdG: 8-hydroxy-2'-deoxyguanosine

ALT: Alanine Aminotransferase

ANOVA: Analysis of Variance

AST: Aspartate Aminotransferase

ATP: Adenosine Triphosphate

BW: Body Weight

CAT: Catalase

CREA: Creatinine

CRE: Creatinine

CYP: Cytochrome P450

D: Day

DCFDA: 2',7'-Dichlorodihydrofluorescein Diacetate

df: Degrees of Freedom

DNA: Deoxyribonucleic Acid

FRAP: Ferric Reducing Antioxidant Power

GC-MS: Gas Chromatography-Mass Spectrometry

GSH: Glutathione

GSH-Px: Glutathione Peroxidase

GSSG: Oxidized Glutathione

GST: Glutathione S-Transferases

H2O2: Hydrogen Peroxide

H2S: Hydrogen Sulfide

HCL: Hydrochloric Acid

HCT: Hematocrit

HGB: Hemoglobin

IC50: Half-Maximal Inhibitory Concentration

IFCC: International Federation of Clinical Chemistry

KCl: Potassium Chloride

kg: Kilogram

KOH: Potassium Hydroxide

LDL: Low-Density Lipoprotein

LD50: 50% Lethal Dose

LDL: Low-Density Lipoprotein

MDA: Malondialdehyde

mm: Millimeters

MLDs: Minimum Lethal Doses

mg: Milligrams

mg/kg: Milligrams per Kilogram

mL: Milliliters

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

nmol: Nanomole

ORAC: Oxygen Radical Absorbance Capacity

QA: Quinolizidine Alkaloids

RCOS: Reactive Carbon Species

ROS: Reactive Oxygen Species

RNS: Reactive Nitrogen Species

RSS: Reactive Sulfur Species

SOD: Superoxide Dismutase

SULTs: Sulfotransferases

spp.: Species

SD: Standard Deviation

TPA: Total Radical-Trapping Antioxidant Parameter

UGTs: UDP-glucuronosyltransferases

URE: Urea

WBC: White Blood Cell

## ABSTRACT

**Mert, G. (2023) Investigation of Repeated Dose Toxicity of Raw and Processed *Lupinus albus* L. Yeditepe University, Institute of Health Science, Department of Pharmaceutical Toxicology PhD thesis, İstanbul.**

**Aim:** The aim of this research is to do a comprehensive toxicological assessment of *Lupinus albus* L. (lupine) products, to prove their safety profile. Lupine is a plant species known for its significant variability in quinolizidine alkaloid (QA) composition, which can be varied not only among different genetic forms within the same species but also in response to various environmental factors such as soil conditions and drought. The potential toxic effects of lupine seeds on vital organs, particularly the liver and kidneys were studied, as well as their impact on oxidative stress markers in a Sprague Dawley rat model. Both debittered and crude lupine seeds were administered at different dosage levels over a 28-day period. The evaluation of organ damage, oxidative stress parameters, and other relevant factors were carried out to provide a comprehensive understanding of lupine toxicity and its implications for food and animal provender safety.

**Materials & Methods:** The Sprague Dawley rat model was used in this study. Rats were divided into 4 groups as low dose (665 mg/kg bw/d) processed (debittered) and crude (unbittered) lupine seeds suspension and high dose (2000 mg/kg bw/d) processed (debittered) and crude (unbittered) lupine seeds suspension with 5 males and 5 females in each group. lupine seeds administered by oral gavage to each group for 28 days. Extensive blood biochemistry analyses were conducted, organ tissues were collected and histopathological examination conducted. Additionally, animal weight gain was monitored, and hemogram tests were conducted to assess overall well-being. Various statistical tests, including ANOVA, Welch ANOVA, Kruskal-Wallis tests, and Bonferroni corrections applied to analyze the data.

**Results:** Distinct patterns of organ damage in both the liver and kidneys are revealed in the study across different experimental groups. Notably, the consumption of crude lupine seeds suspension induced necrosis, infiltration and congestion in these organs, irrespective of dosage or gender. In contrast, processed lupine seeds suspension exhibit no observable damage to the liver and kidneys. Trends in oxidative stress markers highlighted, suggesting a potential reduction in oxidative stress with higher doses of processed lupine. Additionally, a gender-specific response to lupine consumption

observed in white blood cell count. These findings provide valuable insights into lupine toxicity and its potential applications in food and animal provender safety.

**Conclusion:** The consumption of crude (non-debittered) lupine seeds suspension led to necrosis and congestion in both the liver and kidneys, regardless of gender, at both low and high doses. The primary variation in the severity of damage was observed in the infiltration in both organs, with infiltration noted at higher doses. However, the consumption of processed (debittered) lupine seeds suspension at both low and high doses did not negatively impact the normal functioning of the liver and kidneys, irrespective of gender. There was a statistically significant difference in body weight suggest that lupine consumption may affect the growth and development of animals. Furthermore, our finding suggests that lupine consumption may have differential effects on ALT levels and may influence WBC count differently in males and females. Additionally, our observation suggests that lupine may have a dose-dependent effect on oxidative stress markers. On the other hand, negative results were prevented by the traditional debittering methods when correctly applied. These findings provide valuable insights into lupine toxicity and the potential benefits of debittering in reducing adverse effects on vital organs. Further research is warranted to elucidate the underlying mechanisms of lupine toxicity and its safe use.

**Key Words:** "*Lupinus albus* L.", "Lupine toxicity", "Quinolizidine alkaloids", "Rat model", "Subacute toxicity", "Organ damage", "Oxidative stress markers", "Food safety"

# ÖZET

**Mert, G. (2023) Ham ve İşlenmiş *Lupinus albus* L.'nin Tekrarlayan Doz Toksisitesinin İncelenmesi. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmasötik Toksikoloji Anabilim Dalı Doktora Tezi, İstanbul.**

**Amaç:** Bu araştırmanın amacı *Lupinus albus* L. tohumlarının işlem görmeden (ham halde) ve işlem gördükten sonraki güvenlik profillerini kanıtlamak için kapsamlı bir toksikolojik değerlendirme yapmaktır. Termiye, Kinolizidin alkaloit (QA) bileşiminde önemli değişkenliğiyle bilinen bir bitki türüdür; bu değişkenlik, yalnızca aynı tür içindeki farklı genetik formlar arasında değil, aynı zamanda toprak koşulları ve kuraklık gibi çeşitli çevresel faktörlere yanıt olarak da değişiklik gösterebilir. Termiye tohumlarının hayati organlar, özellikle karaciğer ve böbrekler üzerindeki potansiyel toksik etkilerinin yanı sıra oksidatif stres belirteçleri üzerindeki etkileri Sprague Dawley sıçan modelinde araştırıldı. Hem işlenmiş hem de ham termiye tohumları, 28 günlük bir süre boyunca farklı dozaj seviyelerinde uygulandı. Termiye toksisitesinin gıda ve hayvan yemi güvenliği üzerindeki etkilerinin kapsamlı bir şekilde anlaşılmasını sağlamak için organ hasarı, oksidatif stres parametreleri ve diğer ilgili faktörler değerlendirildi.

**Materyal ve Yöntemler:** Bu çalışmada Sprague Dawley sıçan modeli kullanıldı. Sıçanlar her grupta 5 erkek ve 5 dişi olacak şekilde, düşük doz (665 mg/kg vücut ağırlığı/gün) işlenmiş (acılığı giderilmiş) ve ham (acılığı giderilmemiş) termiye süspansiyonu ve yüksek doz (2000 mg/kg vücut ağırlığı/gün) işlenmiş (acılığı giderilmiş) ve ham (acılığı giderilmemiş) termiye süspansiyonu olmak üzere dört gruba ayrıldı. Termiye süspansiyonları her gruba 28 gün boyunca oral gavaj yöntemi ile uygulandı. Kapsamlı kan biyokimyası analizleri yapıldı, organ dokuları toplandı ve histopatolojik inceleme yapıldı. Ek olarak, hayvanların kilo alımı izlendi ve genel refahı değerlendirmek için hemogram testleri yapıldı. Verileri analiz etmek için ANOVA, Welch ANOVA, Kruskal-Wallis testleri ve Bonferroni düzeltmeleri dahil olmak üzere çeşitli istatistiksel testler uygulandı.

**Bulgular:** Farklı deney gruplarında yapılan çalışmada hem karaciğerde hem de böbreklerde farklı organ hasarı modelleri ortaya çıktı. Özellikle, ham termiye tohumunun tüketimi, dozaj veya cinsiyete bakılmaksızın bu organlarda nekroza, infiltrasyona ve tıkanıklığa neden olmuştur. Bunun aksine, işlenmiş termiye süspansiyonu, karaciğer ve böbreklerde gözlemlenebilir bir hasar sergilememektedir. Oksidatif stres belirteçlerindeki

değişimler vurgulanmıştır ve bu durum, yüksek dozda işlenmiş termiye kullanan grupta, oksidatif streste potansiyel bir azalma olduğunu düşündürmektedir. Ek olarak, beyaz kan hücresi sayımında cinsiyete özgü bir yanıt gözlendi. Bu bulgular termiye toksisitesi, gıda ve hayvan yemi güvenliği hakkında değerli bilgiler sağlamaktadır.

**Sonuç:** Ham (acılığı giderilmemiş) termiye tohumlarının tüketimi, cinsiyete bakılmaksızın hem düşük hem de yüksek dozlarda sıçanlarda hem karaciğerde hem de böbreklerde nekroz ve tıkanıklığa yol açmıştır. Hasar şiddetindeki değişiklik, her iki organda da yüksek dozlarda infiltrasyon olarak gözlendi. Bununla birlikte, işlenmiş (acılığı giderilmiş) termiye tohumlarının hem düşük hem de yüksek dozlarda tüketimi, sıçanlarda cinsiyete bakılmaksızın karaciğer ve böbreklerin normal işleyişini olumsuz etkilememiştir. Hayvan ağırlığında istatistiksel olarak anlamlı bir fark bulunması, acı bakla tüketiminin hayvanların büyüme ve gelişmesini etkileyebileceğini düşündürmektedir. Ayrıca bulgumuz; termiye tüketiminin ALT seviyeleri üzerinde farklı etkileri olabileceğini ve erkek ve dişilerde WBC sayısını farklı şekilde etkileyebileceğini göstermektedir. Ek olarak, gözlemlerimiz termiyenin oksidatif stres belirteçleri üzerinde doza bağlı bir etkiye sahip olabileceğini düşündürmektedir. Öte yandan geleneksel acılığı giderme yöntemleri doğru uygulandığında olumsuz sonuçların önüne geçilmiştir. Bu bulgular termiye toksisitesi ve hayati organlar üzerindeki olumsuz etkilerin azaltılmasında acılık gidermenin potansiyel faydaları hakkında değerli bilgiler sağlamaktadır. Termiye toksisitesinin altında yatan mekanizmaları ve güvenli kullanımını aydınlatmak için daha fazla araştırma yapılması gerekmektedir.

**Anahtar Kelimeler:** "*Lupinus albus* L.", "Termiye toksisitesi", "Kinolizidin alkaloit", "Sıçan modeli", "Subakut toksisite", "Organ hasarı", "Oksidatif stres belirteçleri", "Gıda güvenliği"

## 1. INTRODUCTION

Lupine, a variety of leguminous plant, finds frequent use in agriculture for both nutritional and fertilization purposes owing to its nitrogen-fixing capabilities. The seeds of lupine are notably rich in protein, comprising approximately 30-40%, a nutritional profile deemed to surpass that of soybeans. This has prompted discussions about the possibility of introducing lupine as an alternative to soy in human dietary preferences. [1]

In the realm of the *Lupinus* genus, more than four hundred distinct species have been recognized; however, only four among them have been cultivated and harnessed for agricultural and industrial applications. These four are the white lupine (*Lupinus albus)* as shown in Figure 1 below, narrow-leaved lupine (*Lupinus angustifolius*), golden lupine (*Lupinus luteus*), and Andean lupine (*Lupinus mutabilis*). [1,2]

Lupines are predominantly distributed across South and North America, with a limited assortment of species (twelve variations) observed in Europe and North Africa. Within Europe, the primary utilization involves white and golden lupine, serving purposes such as animal feed, fertilization, and human consumption. [3]

The diversity in quinolizidine alkaloids (QAs) content is intricate, as it can differ not only among various genetic variants of the same species but also due to the impact of environmental elements like soil properties and periods of drought. [4,5]

**Figure 1.** Image capturing the appearance of *Lupinus albus*, commonly known as white lupine. [271]

# 2. SOURCE RESEARCH

## 2.1. Lupine General Information

In Türkiye, *Lupinus albus*, commonly referred to as termiye or tırmız, is recognized under various names such as rabies bean, gavur bean, wolf bean, corn bean, Jewish bean, and most frequently, termiye. [6] Beans hold significant prominence as a substantial plant-derived protein source in human diets, especially in developing nations. They often boast considerable fiber and carbohydrate content. Legumes encompass supplementary elements such as lipids, polyphenols, and bioactive peptides. [7,8,9]

Lupine (*Lupinus* spp.) represents an annual herbaceous botanical member of the legume family, scientifically known as Fabaceae or Leguminosae. [8,10,11] The lupine plant achieves a growth stature of approximately one meter. Its foliage consists of segmented leaves adorned with fine hairs. The lupine bears fruits that blossom during June-July and mature in the autumn. These fruits take on a somewhat rounded, dirty yellow appearance. Historical records suggest that ancient Egyptians and Romans incorporated lupine into their diets. [12,13]

Lupine stands as an exceptionally nourishing plant, boasting a protein content twenty-three times greater than cereals, along with an abundance of vitamins, minerals, calcium, and iron. In the realm of protein content, it holds its ground alongside soybeans. [14,15] When juxtaposed with grains, lupine proteins exhibit elevated levels of lysine while maintaining lower quantities of sulfur amino acids. [16] The oil composition within lupine fluctuates within the range of 6-13%, with a pronounced presence of polyunsaturated fatty acids. [17,18]

Lupine seeds also offer a substantial dietary fiber content ~30%, making them a potential source of essential cellulose for nutritional food manufacturing. The growing significance of lupine within the food sector stems from its considerable potential as a food component. [19-22] Lupine, specifically *Lupinus albus* ssp., holds a longstanding and valuable position among legumes. It thrives across diverse soil and climate conditions and boasts a notable protein content, alongside essential dietary minerals. Lupine has historically served as a food source in Mediterranean regions and the elevated terrains of the Andes. [23,24]

The cultivation of lupine is driven by several factors, notably its elevated protein and oil content, rendering it a valuable contributor to human nutrition, animal feed, and

soil enhancement. Additionally, the pharmaceutical sector employs its cultivation as a source of raw materials to investigate the effects of the natural alkaloids and pesticides present within it. [17,25,26] Lupine finds extensive cultivation and usage across a range of countries including Germany, Poland, Portugal, Hungary, Denmark, the Netherlands, France, Italy, Spain, South Africa, New Zealand, South America, among others. It generally exhibits strong adaptability in regions where soybeans, legumes, chickpeas, lentils, and other leguminous crops struggle to thrive. [27]

In the realm of agriculture, only four distinct species from the extensive array of over four hundred known lupine species hold significant importance. These species include *Lupinus albus* (white lupine), *Lupinus angustifolius* (blue or narrow-leaved lupine), *Lupinus luteus* (yellow or golden lupine), and *Lupinus mutabilis* (pearl or Andean Lupin). The Mediterranean region, encompassing countries like Türkiye, serves as the native habitat for the first three species, while *Lupinus mutabilis* originates from South America. [11,28,29]

Lupine cultivation is notably observed within Türkiye 's Central Anatolia region. It also thrives in parts of Konya that serve as an entry point to the Mediterranean region. The total lupine cultivation area in Türkiye spans 3,293 hectares, yielding 356 tons and averaging 108 kg per hectare. The relatively lower production and marketing levels of lupine in Türkiye, when compared to other agricultural commodities, can be attributed to several factors. These include the limited availability of suitable regions that meet the crop's specific climate and soil requirements, a lack of advanced processing and utilization technologies, and inadequate awareness of the crop within the country. [30-33]

Lupine presents a well-balanced carotenoid profile comprising beta-carotene, lutein, zeaxanthin, tocopherols, and other active compounds. The lipid fraction of lupine prominently features lupeol, a triterpene alcohol that plays a role in the remodeling of epidermal tissue. [34-41] This versatile plant, housing alkaloids such as lupanine, sparteine, and anagyrine, holds a significant position within the pharmaceutical industry. Furthermore, lupine and its derivatives serve as components in various products including bread, cookies, cakes, pasta, confectionery, soy sauce, soy substitutes, and vegetable oils. It finds utility as a premium antioxidant-rich gluten-free flour, an emulsifier, a milk alternative, and a snack. [43,43]

Lupine seeds can be categorized as either "sweet" with low alkaloid content or "bitter" with high alkaloid content, determined by their alkaloid levels. The development of "sweet" lupine varieties, which possess minimal or even absent alkaloids, has been achieved through crossbreeding research in countries including Germany. [30,44] However, "bitter" lupine seeds varieties require purification processes to eliminate some of the toxic, bitter alkaloids before they can be considered safe for consumption. [43,45] Alkaloids can induce symptoms such as weakness, breathlessness, convulsions, and severe neurological effects that can ultimately result in respiratory failure and death. [46]

Numerous straightforward technological, chemical, and traditional techniques, as in Figure 2 below, are available to mitigate the bitterness in lupine seeds. These methods encompass approaches such as boiling, cold-water immersion, rinsing under running water, autoclaving, and treatment with solutions of saline (HCl) and potassium hydroxide (KOH). [30,44] Traditional methods are employed to purify the seeds from alkaloids. One such method involves boiling the seeds in warm water maintained at temperatures of 60-70°C for a duration of 12 hours. Subsequently, the seeds are placed in bags and subjected to a process of water change, with at least 4/5 water changes occurring over a 2 to 4-day period within stagnant water in personal reservoirs. [28,43]

**Figure 2.** Image of lupine seeds soaking in clear water using a traditional technique to eliminate bitterness.

Lupine serves as a promising substitute for soy protein due to its comparable amino acid composition and higher protein content. Alongside lupanine and albine, it contains other alkaloids like sparteine and anagyrine, augmenting its significance within the pharmaceutical realm. [32] White lupine seeds are abundant in minerals, amino acids, omega-6 and omega-3 fatty acids, oligosaccharides, and antioxidants, establishing them as a wholesome dietary choice for humans. [43] These seeds are believed to offer advantages for insulin resistance, diabetes, as well as conditions like heart disease, high blood pressure, and liver health. However, excessive consumption of lupine nuts, particularly in the presence of compounds like lupanine and sparteine, can lead to adverse neurological effects such as dry mouth, tremors, and vision disturbances. [47]

Lupine exhibits potential for use in organic agriculture due to its adaptability to low pH soils. Additionally, the utilization of water filtered and boiled as part of the sweetening process has potential as a natural pesticide for insect management. [48]

### 2.1.1. Fabaceae (Leguminosae)

Lupine resides within the plant kingdom and falls under the division of seeds plants. It is categorized within the subclass of flowering plants, belonging to the class of dicotyledons. It finds its place in the order of Fabales, within the family Fabaceae, specifically the subfamily of Faboideae. More precisely, it belongs to the subtribe of *Lupanine*. Taxonomically, it is situated within the genus *Lupinus*. Members of this family can exhibit either woody or herbaceous characteristics, featuring alternate leaves that are typically smooth, compound, and bipinnate or triple. The flowers display a characteristic of being either zygomorphic or actinomorphic, frequently being hermaphroditic and arranged in racemose clusters. These flowers generally possess four or five sepals, with one often positioned at the forefront. Characteristics of *Fabaceae* family shown in Figure 3 below, the petals can either be valvate or imbricate, occasionally fused partially or absent. The stamens can be unisexual and either free or fused. The fruit characteristic of this family is a legume, commonly known as a bean, containing one or more seeds. [49,50]

The Fabaceae family is subdivided into three distinct subfamilies. The Mimosoideae subfamily encompasses woody trees, shrubs, and herbaceous plants characterized by compound, feathery leaves. Their small, vibrant flowers are arranged in spike-like or pointed inflorescences. The Caesalpinioideae subfamily consists of trees,

shrubs, or occasionally herbaceous plants that feature hairy leaves and lively flowers. The Faboideae subfamily, also referred to as Papilionaceae or Papilionatae, is primarily composed of herbaceous, shrubby, or semi-shrubby plants, and occasionally includes trees. These plants typically bear clover-like, pubescent, simple, or elliptical leaves. Flowers within this subfamily are typically zygomorphic, though they can sometimes be actinomorphic, arranged in panicle-like inflorescences. Among its distinctive features, three of the petals are compound, with the upper petal being large and erect. The two smaller petals located on the sides function as wings, while the lower petals fuse together to form the keel. [51,52]

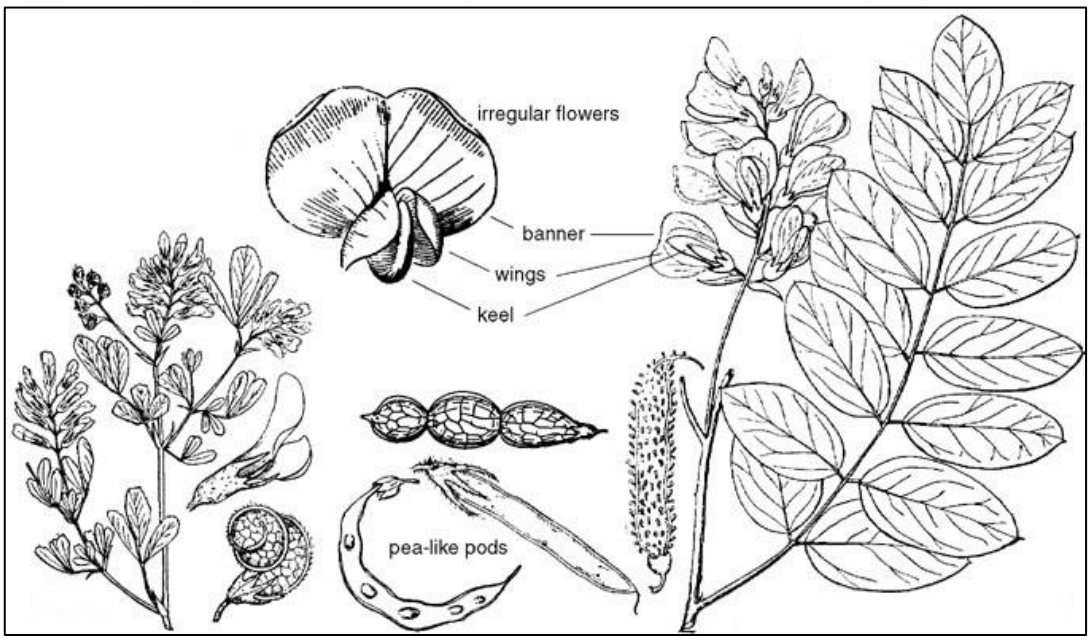



**Figure 3.** Characteristics of the family of legumes: *Fabaceae*. [272]

### 2.1.1.1. *Lupinus* L. Species Distributed in Türkiye

Within the *Lupinus* L. genus, there are eight distinct taxa spanning across six species that can be identified in Türkiye, as shown in Figure 4 below. These species include *L. angustifolius*, *L. angustifolius* subspecies reticulatus, *L. albus*, *L. albus* subspecies graecus, *L. micranthus*, *L. hispanicus*, *L. luteus*, and *L. anatomicus*. [50]

*Lupinus albus* is an annual plant that can attain a height of 120 cm. Its leaves consist of ovate-shaped leaflets measuring 25-35 x 14-18 mm, displaying a mucous texture and being covered in hair on both sides. The inflorescence, positioned alternately, spans 15-16 mm. The calyx, measuring 8-9 mm, is divided into lobes. The corolla, ranging from 15 to 16 mm, exhibits color variations between white and blue. The seeds have a smooth surface and measure 8-14 mm in size. Lupine engages in a flowering period lasting 5-6 months. [50]

*Lupinus albus* subspecies graecus represents a particular variant within the lupine plant. It is characterized by a corolla that is entirely blue in color. Its legume measures approximately 60-70 mm in length and 11-13 mm in width, enclosing seeds that are about 8-9 mm in size. [50]

*Lupinus angustifolius* is a short-haired annual plant that reaches heights ranging from 20 to 80 cm. Its leaflets are linear or spatulate, measuring 20-50 mm in length and 2-4 mm in width. The flowers are arranged alternately, and the corolla spans 11-13 mm, displaying a blue hue. The calyx features a two-part upper lip, and the legume is short, measuring 8-13 mm, and covered in hair. The color of the legume varies from yellow to black. Within the legume, the seeds, which measure 6-7 mm, are flat and come in assorted colors. [53]

*Lupinus albus* subspecies *reticulatus* is an annual plant characterized by short hair, reaching a height of 20-40 cm. Its leaflets measure 10-20 mm in length and 2 mm in width, displaying variations in thickness and straightness. The legume is sized between 35-45 mm in length and 6-8 mm in width, while the seeds are about 4.5-5 mm in length and 3-3.5 mm in width. [54]

*Lupinus albus* subspecies *micranthus* exhibits a height range of 10 to 40 cm. Its leaves are oval-shaped and mucilaginous, measuring 15 to 70 mm in length and 5 to 15 mm in width. The flowers reach lengths of 5 to 12 cm and display dark lips divided into two parts, alongside a dark bottom lip divided into three sections. The corolla is blue, measuring 10 to 14 mm in length. [55]

*Lupinus albus hispanicus* features leaflets that are sized 40-60 mm x 8-12 mm, taking on an ovoid or rectangular form. Its racemes extend between 5 to 16 cm in length, while its flowers are meticulously arranged. The corolla measures 13-16 mm, initially appearing cream in color and gradually transitioning to shades of red and purple as it matures. The calyx's upper lip is divided into two segments. The legumes exhibit

dimensions of 40-50 mm x 10 mm and are adorned with short hair, displaying a color spectrum from green to black. Seeds, measuring 4.5-6 mm, possess a tuberous shape and exhibit a multi-colored appearance. [55]

*Lupinus albus luteus* can attain heights ranging from 20 to 80 cm. Its leaves encompass ovate or oblong leaflets, measuring 30-60 mm in length and 8-15 mm in width. The flowers are organized in a raceme and extend 5-25 cm in length. The flower stalk is concise, and the calyx features an upper lip that is deeply divided, spanning 6-7 mm, along with a lower lip bearing three teeth that measures 10 mm. The corolla exhibits a golden yellow hue and measures 14-16 mm in length. The legumes showcase ribs, measuring 40-60 mm in length and 10-14 mm in width. The plant yields 4-6 seeds, each measuring 6-8 mm in length and appearing flat. [56]

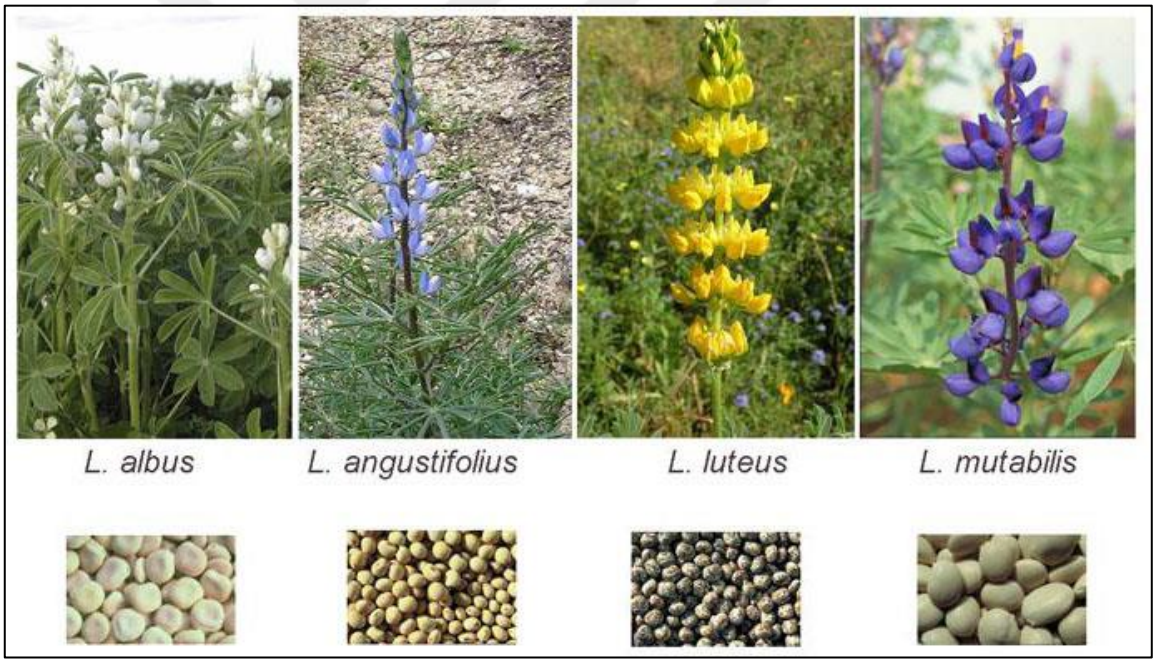



**Figure 4.** Various species of the *Lupinus* L. genus found in Türkiye. [285]

## 2.2. Lupine Chemical Composition

Lupine, a member of the Fabaceae family, contains a diverse array of nutrients and bioactive compounds. The lupine grain possesses approximately 7% water content, with an ash content that varies between 1.47% and 3.4%. Its crude protein content ranges from 32.0% to 36.7%, comprising 12.8% albumin and 79.2% globulin. Additionally, lupine contains an oil content that falls within the range of 11.5% to 15.1%, crude fiber content that spans from 9.8% to 16.2%, and modest amounts of thiamine, riboflavin, and niacin. [60,61,62] The oil content in lupine can vary depending on the genotype and extraction method, falling into ranges of 5-10%, 8-21%, or 19.4%. The majority of lupine oil consists of unsaturated fatty acids, with an approximate ratio of 90% polyunsaturated to 10% saturated fatty acids, as indicated by sources. [18,39,57,63] Within the unsaturated fatty acids, lupine oil comprises 32-50% oleic acid, 17-47% linoleic acid, 3-11% linolenic acid, 11.6% palmitic acid, and 1.9% stearic acid. Notably, lupine oil exhibits a higher omega-3 to omega-6 fatty acid ratio compared to most vegetable oils and is additionally rich in tocopherols and phenolic compounds. [64-66]

Lupine contains various anti-nutritional components, including trypsin inhibitors, tannins, saponins, lectins, and phytates, which have the potential to adversely affect digestion. However, it's important to note that the concentrations of these factors are generally low. Lupine also contains quinolizidine alkaloids, such as lupanine, lupine, sparteine, spatulatine, hydroxylupanine, monolupine, puziline, angustifoline, lupinyl glycosides, and vernine, with levels that can range from 0.5% to 6%. Notably, these alkaloid levels are significantly lower in sweet lupine, typically falling within the range of 0.008% to 0.012% [70-72]

Lupine is rich in dietary fiber, comprising approximately 34.44% to 39.42% of its overall composition. Within this, around 3.64% to 5.21% is soluble fiber, while the majority, approximately 30.80% to 34.22%, is insoluble fiber. [65,73] Additionally, lupine exhibits an impressive 8-fold water-absorbing capacity. The primary constituents of its insoluble dietary fiber include cellulose, constituting 79% of the fiber, followed by hemicellulose at 14%, and lignin at 7%. [74] Lupine is characterized by its low starch content, which can range from 0% to 5%, and its comparatively higher levels of non-starch polysaccharides, typically ranging from 30% to 40%. [75] Non-starch polysaccharides found in lupine can be broken down without causing lactic acid accumulation. [17,77]

The primary monosaccharides present in lupine include galactose, arabinose, and uronic acid, all of which are constituents of reserve pectic substances. Lupine also contains sucrose in the range of 1.5% to 3.5%, stachyose at 6% to 7.5%, raffinose ranging from 0.5% to 0.9%, and verbascose oligosaccharides at levels of 0.3% to 0.8%. In total, lupine seeds contain approximately 7.4% to 8.0% oligosaccharides. [17,77]

Lupine flour has been shown to contain significant amounts of various minerals, as indicated by studies conducted by Yorgancılar et al. and Bilgiçli and Levent. The specific mineral levels in lupine flour were reported as follows: 396 mg/100 g for calcium, 100.5 mg/100 g for magnesium, 152.89 mg/100 g for manganese, 1.79 mg/100 g for copper, 4.62 mg/100 g for iron, 59.2 mg/100 g for potassium, 615.5 mg/100 g for phosphorus, and 5.29 mg/100 g for zinc. [6,78]

## 2.3. Quinolizidine Alkaloids

Alkaloids are intricate chemical compounds generated by organisms as a metabolic byproduct. Alkaloids are capable of dissolving in acidic water solutions as well as various organic solvents like ethers, chloroform, and ethanol. [79-80] The term "alkaloid" derives from the word "alkali," signifying a base, and the suffix "oid," which means resembling. This nomenclature is attributed to the fact that alkaloids exhibit varying degrees of basicity depending on their molecular structure, the presence of specific functional groups, and their respective positions within the molecule. [88]

Quinolizidine alkaloids (QAs), shown in Figure 5 below, are chemical compounds that plants manufacture as a means of defense against potential threats such as herbivores and pathogenic microorganisms. [1,81-84]

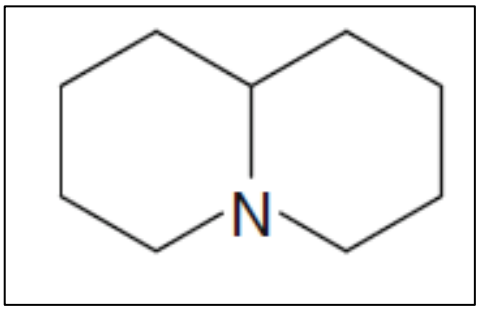



**Figure 5.** An illustration depicting the Quinolizidine ring. [84]

Alkaloids represent a class of naturally-occurring nitrogen-containing chemical compounds. These bioactive substances encompass a wide spectrum, ranging from uncomplicated amines to intricate cyclic structures. The categorization of alkaloids hinges on the existence of a CN skeleton. [80] Certain alkaloids derived from plants exhibit distinctive characteristics. These compounds, integral to plant defense mechanisms, can also double as stores of energy. Alkaloids are known for their bitter and alkaline taste and can exert noteworthy influences on the nervous systems of organisms. They can be readily extracted and isolated for study and use. [84]

Alkaloids are natural secondary metabolites that occur in a wide range of sources, including plants, animals, microorganisms, and fungi. They are typically identified by their cyclic structure, which contains one or more nitrogen atoms. While alkaloids are predominantly found in plants, they share common chemical properties. They are also highly pharmacologically active, even at relatively low doses. The classification of alkaloids is based on their biosynthetic pathways, which are determined by the amino acids used by plants as raw materials for their production. For instance, alkaloids originating from ornithine are referred to as tropanic alkaloids. [85-87].

### 2.3.1. Lupine Alkaloids

Wild tropical plants can possess alkaloid concentrations exceeding 10,000 mg/kg. The levels of alkaloids in various parts of the plant, such as leaves, roots, and stems, undergo fluctuations as the plant matures, peaking during the flowering phase. Many of these compounds contain significant or altered quinolizidines. [84]

Numerous alkaloids have the potential to induce cramps, vomiting, and in severe cases, even death by causing respiratory paralysis. When taken in substantial amounts, they can also produce adverse effects on the central nervous system in mammals, albeit typically of mild nature. In humans, extremely high doses of specific alkaloids like lupanine and sparteine may lead to symptoms such as tremors, euphoria, convulsions, blurred vision, dry mouth, irritability, and a low mood. [47,90] The main alkaloids in most lupine species are tetracyclic quinolizidine alkaloids (QAs), which include sparteine, lupanine, and 13αhydroxylupanine, varieties are shown in Figure 6 and 7 below. [4,82]

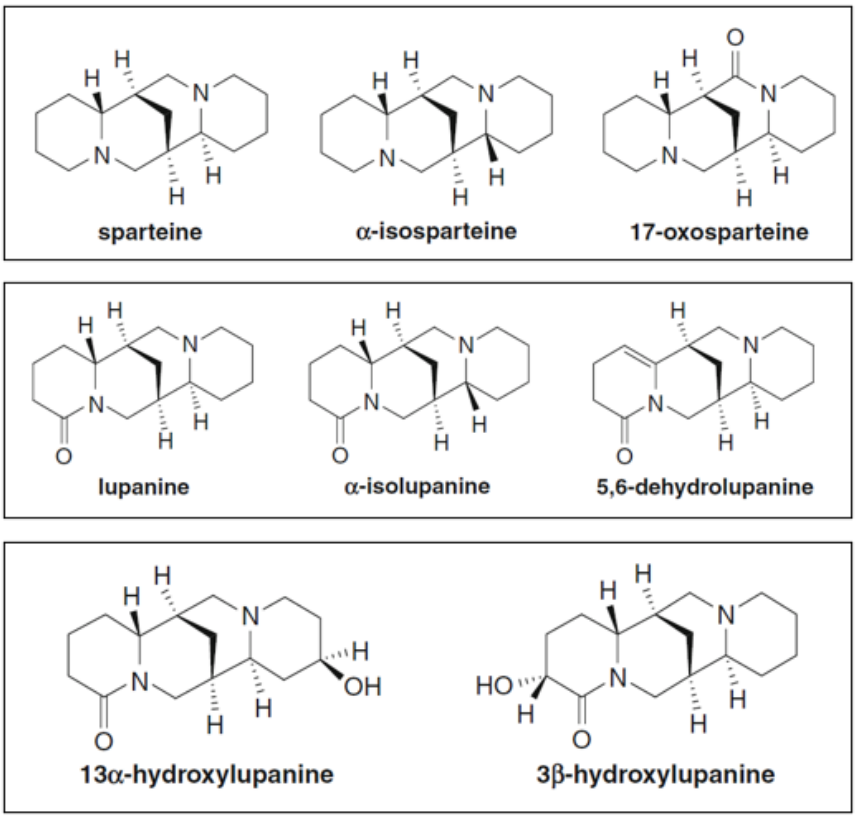



**Figure 6.** Varieties of Quinolizidine alkaloids (QAs). [84]

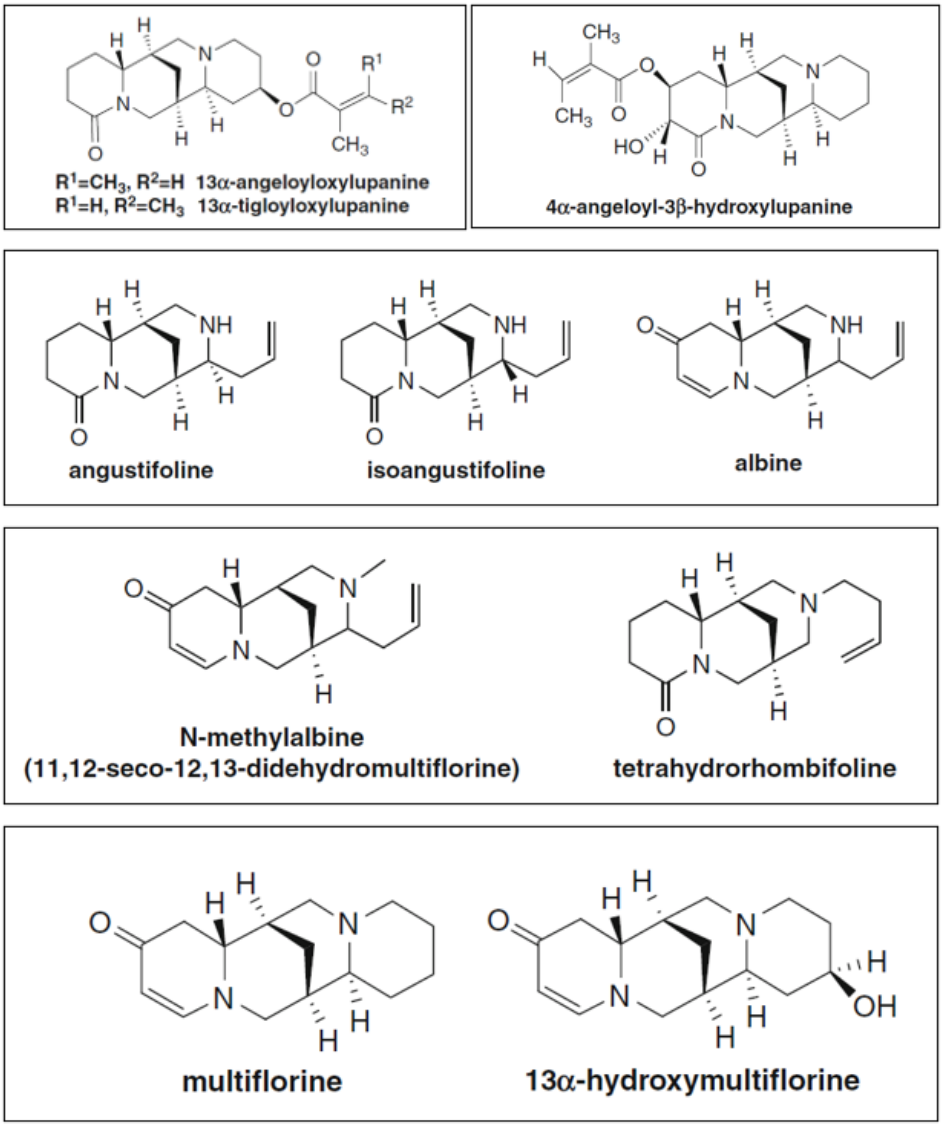



**Figure 7.** Varieties of Quinolizidine alkaloids (QAs) (continues). [84]

## 2.4. Lupine Usage

Research findings have indicated that the lupine plant holds significant promise as a valuable reservoir of dietary nutrients, thereby piquing the interest of the food industry. [19,21] It has been proposed as a viable source for the production of nutritious foods. [65] Furthermore, in certain countries, lupine is utilized as an additive in a diverse

array of products. These include frozen dairy desserts, jams, dairy items, baked goods, puddings, and meat products. [21]

Lupine seeds offer a valuable vegetable protein source that can serve as a substitute for soybean meal in broiler chicken feed. The growing prevalence of genetically modified soybeans has generated a desire for natural and alternative plant protein sources in recent years. When formulating diets for broiler chickens, it becomes crucial to take into account factors like digestible amino acid levels, nonstructural polysaccharides in the cellular wall, and alkaloid content present in lupine seeds. These considerations point toward an increased demand for lupine seeds, spanning both traditional and organic farming operations. [91]

Sparteine, typically in the form of sparteine sulfate, has found medical applications as a treatment for heart arrhythmias and as a means to stimulate uterine contractions during childbirth. Additionally, it has served as a marker for assessing the metabolic activity of cytochrome P450 (CYP) 2D6. [92]

### 2.4.1. Lupine Effect on Human Health

High protein diets play a crucial role in supporting the growth and recuperation of adolescents and athletes, as well as during the phases of pregnancy and lactation. Certain lupine proteins, or their components, hold the potential to offer beneficial effects on the human body in various challenging conditions, including diabetes, hypertension, obesity, and cardiovascular ailments. These proteins may also aid in regulating blood sugar levels among individuals with diabetes. Research has provided evidence that the consumption of lupine protein-rich foods can lead to significant reductions in serum cholesterol levels, encompassing LDL (low-density lipoprotein, or "bad" cholesterol) and triglycerides, along with improvements in blood sugar and blood pressure. [84]

These alkaloids exhibit diuretic properties, which means they can promote the production of urine, potentially aiding in the reduction of kidney stones and sand while clearing the urinary tract. Additionally, they might offer relief from conditions like rheumatism, sciatica, and headaches. Furthermore, these alkaloids may assist in eliminating excess salt from the body. [93] Lupine, characterized by its very low glycemic index owing to its minimal starch content, has been employed in diabetes management [94]. Research indicates that it can have favorable impacts on conditions such as obesity, hypertension, elevated cholesterol levels, and insulin resistance. Additionally, it may

contribute to appetite suppression and maintaining energy balance [96]. Despite its high nutritional value and health benefits, lupine remains underutilized as a source of nutrition and does not receive the attention it deserves [39].

## 2.5. Lupine and Alkaloids Toxicology

The majority of research concerning lupine alkaloids has concentrated on sparteine and lupanine. Both of these alkaloids exhibit a low level of acute toxicity, with sparteine being the more toxic of the two. There is evidence suggesting that these alkaloids can lead to neurological issues, primarily characterized by a loss of motor coordination and muscle control. Importantly, these effects are typically reversible [96-98]. Lupanine and several other lupine alkaloids exhibit moderate toxicity in vertebrates. In mammals, acute poisoning with sparteine, lupanine, or angustifoline can result in symptoms such as convulsions, tremors, and respiratory as well as cardiac failure [99].

### 2.5.1. Mechanism of Action

Lupine alkaloids have been observed to inhibit acetylcholine activity at both nicotinic and muscarinic receptors in pig brain cells in vitro. The inhibitory concentrations, represented by IC50 values, for acetylcholine nicotinic receptors were determined as follows: lupanine at 5 micromolar (μM), 193 μM for sparteine, 310 μM for tetrahydrorhombifoline, 331 μM for sparteine, and more than 500 μM for lupanine and multiflorin. As for acetylcholine muscarinic receptors, the IC50 values were found to be 21 μM for sparteine, 33 μM for albine, 47 μM for multiflorin, 75 μM for 3-hydroxylupanine, 114 μM for lupanine, and 40-100 μM for 13α-hydroxylupanine and lupanine [100].

Sparteine functions by inhibiting sodium channels and reducing potassium permeability in both neurons and pancreatic cells. Lupine alkaloids have the potential to influence uterine contractions when studied in vitro. When examining the impact on an isolated rabbit uterus, lupanine exhibited the most significant effect at a concentration of 1/5, while lupanine dihydrochloride, at a concentration of 1/15, matched the strength of sparteine sulfate. Furthermore, lupine alkaloids possess antiarrhythmic properties that target coronary vessels located distal to the heart. These effects are achieved by eliminating atrial and ventricular fibrillation through the slowing of conduction. The hierarchy of decreasing antiarrhythmic effects among these substances is as follows: sparteine, lupanine, and 13-hydroxylupanine [101].

### 2.5.2. Toxicokinetic and Metabolism

#### 2.5.2.1. Sparteine

The metabolism of sparteine in mice has been investigated following intravenous or gavage administration (at a dose of 50 mg/kg body weight, equivalent to 27.7 mg/kg body weight of sparteine). In mice, no detectable traces of sparteine were found after 3 hours, but approximately 25% of the administered dose was identified as a sparteine metabolite. Additional mouse studies revealed that sparteine undergoes oxidation to lupanine, as confirmed by chromatographic analysis and standard assays. When mice were administered a 50 mg/kg body weight dose of sparteine via intraperitoneal injection, both lupanine and 2,3-dehydrolupanine were detected in their urine, with the highest concentration of lupanine observed at a dose of 16 mg/kg body weight. [104]

Furthermore, pretreatment of mice with CYP inhibitors, such as SKF 525A, led to a reduction in the amount of lupanine in their urine, indicating the involvement of CYP enzymes in the formation of lupanine. Additionally, pretreatment with disulfiram, an inhibitor of aldehyde dehydrogenase (a cytosolic enzyme) and CYP2E1, also inhibited the conversion of sparteine to lupanine. This suggests that the transformation of sparteine into lupanine may entail intermediate products and could potentially involve both microsomal and cytosolic enzymes in the process. [104]

In humans, when sparteine sulfate is administered orally at a dose of 200 mg (equivalent to 110.8 mg of sparteine base, assuming the use of sparteine sulfate as a pentahydrate), it is rapidly absorbed, with approximately 70% of the substance being absorbed into the bloodstream. The highest concentrations of sparteine in the plasma are typically reached around 45 minutes after ingestion. [105]

Both in rats and humans, the elimination of sparteine from the body follows a similar pattern, with a half-life of approximately 2 hours. When sparteine sulfate is administered intravenously to humans at a dose of 100 mg, roughly 35% of the administered dose is excreted unchanged in the urine within a 24-hour period. [105]

Sparteine undergoes metabolism primarily by the enzyme CYP2D6, yielding two main metabolites: 2,3-dehydrosparteine and 5,6-dehydrosparteine, with 2,3-dehydrosparteine being the predominant metabolite [106]. The presence of various polymorphisms in CYP2D6 gives rise to different phenotypes among individuals, including sparteine poor metabolizers (PMs) found in 5-10% of Caucasians and extensive

metabolizers (EMs). Another subtype, known as ultrafast metabolizers of sparteine, is identified in 1-2% of the Scandinavian population, but it occurs more frequently in the Hispanic population (8%) and Ethiopia (29%) [107-109]. The PMs phenotype significantly impacts the elimination of sparteine, as it is primarily excreted unchanged due to low renal excretion in this group. In contrast, EMs eliminate sparteine mostly in the form of metabolites. [107].

#### 2.5.2.2. Lupanine

In experiments conducted on Wistar rats, lupanine in the form of lupanine hydrochloride was administered as part of their diet. In the initial trial, a diet containing 3.4 mg of lupanine per gram (dry weight) was used as the sole component of the diet, which amounted to approximately 23.2-33.1 mg per rat based on their body weight. During the first day of this trial, around 60% of the lupanine dose was excreted in the urine. [111].

In a more extended 29-day trial, rats were given increasing amounts of lupanine in their diet over time. This regimen involved 4 days with 1 mg/g lupanine, followed by 4 days with 3 mg/g, then 4 days with 6 mg/g, and finally 4 days with 9 mg/g lupanine in their diet. Throughout this trial, approximately 70-80% of the lupanine ingested was excreted, with 50-70% found in the urine and 10-14% in the feces. Approximately half of the total amount of lupanine excreted remained unchanged. Importantly, no accumulation of lupanine was observed in the rats over the course of the trial [111].

The metabolism and elimination of lupanine, as shown in Figure 8 below, and 13-α-OH-lupanine were investigated in human subjects, specifically comparing CYP2D6 poor metabolizers (PMs, N=4) and extensive metabolizers (EMs, N=7). These compounds, administered at a dose of 10 mg each, were given to the subjects in a gelatin tablet form along with 700 mL of water on an empty stomach. The study followed a randomized crossover design, with a two-week washout period between the administration of the two compounds. [112]

Both lupanine and 13-α-OH-lupanine were rapidly excreted in the urine, with an average recovery rate of 89-103% within 72 hours. Notably, there was no significant difference in excretion between PMs and EMs. The mean urinary half-life for these compounds was approximately 6 hours, with individual compounds and groups exhibiting a range from 3 to 11 hours. [112]

Lupanine was primarily excreted unchanged in the urine, with only minimal traces of 13-OH-lupanin detected. Interestingly, most of the subjects excreted 13-α-OH-lupanine in its unchanged form, although in some cases, a portion of it (approximately 14-34%) was excreted as lupanine. [112]

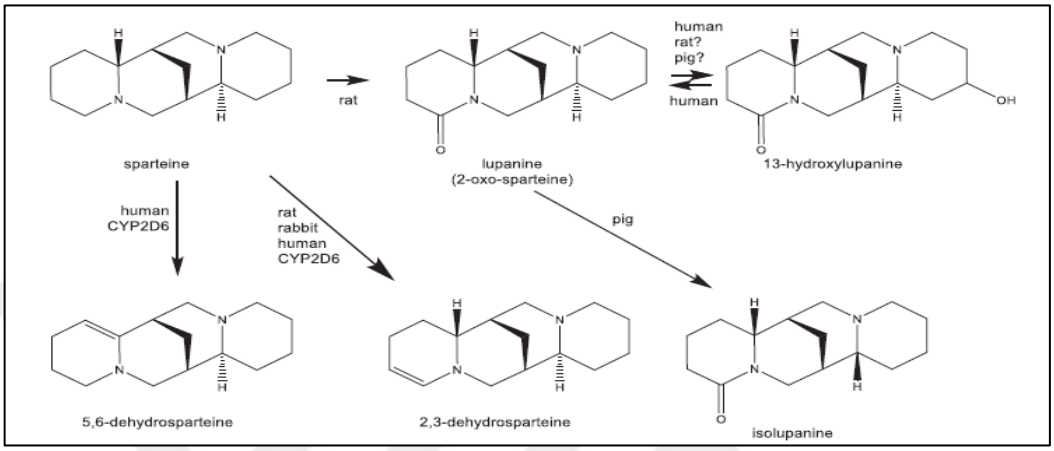



**Figure 8.** The metabolic conversion of sparteine and lupanine in humans, rats, pigs, and rabbits. [113]

### 2.5.3. Acute Toxicity

D-lupanine, an isolated compound derived from *L. angustifolius* seeds and purified via recrystallization, was administered intraperitoneally to mice, rats, and guinea pigs at concentrations of 15 and 100 mg/mL. The minimum lethal doses (MLDs) were found to be 75-85 mg/kg for mice, 180-192 mg/kg for rats, and 210-225 mg/kg for guinea pigs. The 50% lethal dose (LD50) for rats was determined to be 80 mg/kg. Animals receiving a lethal dose of the compound displayed symptoms of paralysis and intermittent convulsive movements, ultimately leading to complete exhaustion. Post-mortem examinations indicated that death resulted from asphyxia, with no evidence of liver damage or other pathological abnormalities observed. It is suggested that the animals succumbed to respiratory failure [144].

Lupanine and sparteine, both derived from *L. albus* seeds and subjected to purification, underwent toxicity assessments in male Swiss rats through oral ingestion

and injection. The oral LD50 for lupanine was determined to be 410 mg/kg body weight, while the LD50 for sparteine was 220 mg/kg body weight. In cases of injection, the LD50 for lupanine was found to be 175 mg/kg body weight, whereas for sparteine, it was 36 mg/kg body weight. Both alkaloids induced similar symptoms, including tremors and seizures, leading to death primarily due to respiratory arrest. [96]

The lower toxicity of lupanine, in comparison to sparteine, was also observed in guinea pigs when administered intravenously, with minimum lethal doses (MLDs) of 78 mg/kg body weight for lupanine and 27 mg/kg body weight for sparteine. The authors of the study propose that the variation in toxicity might be attributed to differences in bioavailability or pharmacokinetics. [96]

The oral LD50 of an alkaloid extract derived from *L. angustifolius* seeds, which contains 49% lupanine, 39% 13-α-OH-lupanine, 10% angustifoline, and 0.7% α-isolupanine, was determined to be 2279 mg/kg in rats that were fed the extract and 2401 mg/kg in fasting rats. In separate experiments, purified lupanine and 13-α-OH-lupanine had oral LD50 values of 1664 mg/kg and 177 mg/kg, respectively, when administered to rats. In the case of mice, the LD50 values for lupanine and 13-α-OH-lupanine were 199 mg/kg and 177 mg/kg, respectively. [97]

When rats were subjected to lethal doses of these alkaloids, they exhibited symptoms such as tremors, convulsions, cyanosis, and lethargy, with death occurring within a relatively short time frame, ranging from 2 to 23 minutes. Post-mortem examinations revealed liver and lung damage across multiple species. However, rats that survived exposure to these alkaloids showed no signs of ongoing clinical toxicity. [97]

In male and female rats, *L. angustifolius* and *L. albus* seeds extracts, containing 10% alkaloids in dry form, were administered through gavage. No fatalities were observed, and the LD50 of these lupine extracts was determined to exceed 4000 mg/kg body weight, which is equivalent to more than 400 mg of alkaloids per kilogram of body weight. [98]

### 2.5.4. Repeated Dose Toxicity

In a 112-day feeding experiment, rats were divided into three groups. The first group received a diet containing casein (referred to as the control group), while the second and third groups were given diets consisting of *L. albus* seeds and *L. luteus* seeds,

respectively. The *L. albus* seeds diet contained 510 mg of lupine alkaloids per kg of food, and the *L. luteus* seeds diet contained 910 mg of lupine alkaloids per kg of food. [115]

Notably, the rats that consumed the lupine seeds diets exhibited lower weight gain in comparison to those in the control group. However, it is worth mentioning that when the protein content of the lupine seeds diets was elevated to 20% and supplemented with DL-methionine, the weight gain of the group fed with *L. luteus* seeds became comparable to that of the control group. [115]

Importantly, there were no significant variations observed in the organ-to-weight ratios or the results of the histopathological examination of the liver, kidney, and lung when comparing the three groups. [115]

During a 12-week investigation, the effects of a diet incorporating *L. mutabilis* flour, containing relatively small quantities of lupine alkaloids (measuring at 160 mg/kg or 14.4 mg/kg body weight per day), were assessed in Sprague-Dawley rats aged 21 days. In this study, the control group was provided with a diet consisting of 15% protein and casein, whereas the experimental group's diet comprised 15% protein with an additional 0.2% DL-methionine. [116]

Notably, the rats that consumed the lupine-based diet did not exhibit any statistically significant differences in terms of growth parameters, including body weight and food intake, when compared to the control group. Furthermore, there were no notable distinctions in physiological factors, encompassing hematological and biochemical parameters. Additionally, the absolute and relative weights of various organs, including the liver, kidney, heart, spleen, and brain, as well as the results of histopathological examinations, did not demonstrate any discernible impact due to the presence of alkaloids. [116]

In a study involving Sprague-Dawley rats, three separate groups were provided with a diet that included *L. angustifolius* flour and was supplemented with *L. angustifolius* seeds extract at varying concentrations: 250 mg/kg, 1050 mg/kg, and 5050 mg/kg of alkaloids. The reference group was given a diet consisting of lupine wheat flour containing 50 mg/kg of alkaloids. [117]

Throughout the course of the study, no fatalities were recorded. However, a marginal reduction in body weight was observed in the group of male rats subjected to the 250 mg/kg diet. Hematological assessments did not reveal any significant effects, and

clinical chemistry examinations showed no discernible distinctions between the rats fed lupine-containing diets and the control group. [117]

Interestingly, in female rats across all doses, there was an increase in the relative weight of the liver. Moreover, some rats in the high-dose group displayed instances of liver parenchymal cell changes. [117]

In a study, rats were subjected to diets containing varying doses of lupine alkaloids (0, 10, 33, 100, or 500 mg/kg body weight per day) for a duration of 90 days. The lupine alkaloid composition encompassed lupanine, 13-α-OH-lupanine, angustifoline, and α-isolupanine. These diets were supplemented with 0.3% methionine. The control diets consisted of commercial formulations, both with and without 4.5% maltodextrin. [117]

Notably, the rats fed the 100 mg/kg/day or 500 mg/kg/day diets exhibited lower body weights in comparison to the control groups. Some variations in hematological parameters and clinical chemistry results were observed, but these deviations remained within normal limits, contingent upon the age and gender of the animals. [117]

Additionally, in rats receiving the 500 mg/kg/day dose, the liver weight was notably higher, with an increase of 8-9% in both males and females compared to the control group. In contrast, the renal weights of the rats displayed inconsistent variations across different doses and were unrelated to gender. [117]

### 2.5.5. Developmental and Teratogenic Endpoint

In a nine-month study, researchers conducted a multigenerational trial using Wistar rats to investigate the effects of a lupine-based diet. The lupine diet consisted of 51.8 grams of *L. albus* powder per 100 grams of the diet and was supplemented with 0.2% DL-methionine. This lupine diet was compared to a control diet with the same protein content (20%), which was made from defatted soybean meal, fish meal, and skimmed milk powder. The total dietary alkaloid level tested was 250 mg/kg, equivalent to 12.5 mg/kg body weight per day. It's worth noting that results for the F0 generation had been reported in a previous study. [118, 119]

In the F1 and F2 generations (offspring of the F0 parent generation mated at 12 weeks of age), the rats received the same lupine diet as their parents. In the male rats of the F1 and F2 generations, the growth rate was higher in the lupine-fed group compared to the control group. However, no significant differences in growth were observed in female rats. There were no notable changes in the relative weights of the heart, spleen,

kidney, brain, and gonads, except for the relative liver weights, which were significantly lower in both male and female rats fed the lupine diet when compared to those fed the control diet. Importantly, histological examinations of the liver and other organs remained normal. [118, 119]

Furthermore, no treatment-related hematological changes or alterations in alanine transaminase (ALT) and aspartate transaminase (AST) levels were observed in either generation. Reproductive performance was also unaffected by the lupine-containing diet. The authors suggested that the difference in relative liver weight between male lupine-fed rats and the control group may have been due to an unusually high liver weight in the control group, which was not observed in previous studies using the same group and a casein diet as the control. [118, 119]

In a study, quinolizidine alkaloids (QAs) extracted from the rhizomes of blue cohosh (*Caulophyllum thalictroides*) were examined for their effects on mouse embryo development in vitro. These QAs were purified and their composition analyzed using gas chromatography-mass spectrometry (GC-MS). The research aimed to assess the overall development and morphology of rodent embryos during the organogenesis stage. Mouse embryos were removed from their mothers at 9.5 days of gestation and cultured with the test compounds for 45 hours. [120]

Two specific QAs, namely α-isolupanine and N-methylcytisine, which were isolated from the extracts (though purity was not specified), were tested for their effects. α-isolupanine exhibited no activity at any of the tested concentrations (5, 20 μg/mL). However, N-methylcytisine, tested at concentrations of 5, 20, 50, 80, and 250 μg/mL, induced significant malformations in the embryos, including anterior neural tube regurgitation, ocular development abnormalities, and torsion of the tail, particularly at the 20 μg/mL concentration. [120]

Anagyrine, another QA, was also examined at concentrations of 5, 20, and 250 μg/mL. While it inhibited normal development at the highest concentration tested, it did not induce teratogenic effects. It's important to note that bone growth was not recorded in this trial, and the study primarily focused on organ development and morphology. [120]

### 2.5.6. Genotoxicity

In one study, several quinolizidine alkaloids, including anagyrine, angustifoline, cytisine, 3-β-OH-lupanine, 13-α-OH-lupanine, lupanine, 17-oxosparteine, sparteine, and

13-α-tigloyloxylupanine, were investigated for their interaction with DNA. The results indicated that these alkaloids did not exhibit DNA binding or intercalation activity. This conclusion was drawn based on assessments such as methyl green DNA release from DNA and the inhibition of DNA polymerase I [1].

In another study, it was specifically observed that cytisine, lupanine, and sparteine, which belong to the quinolizidine alkaloid class, did not bind to or intercalate with DNA. This set them apart from other types of alkaloids like indole and quinoline alkaloids, which can interact with DNA. Furthermore, these quinolizidine alkaloids were found not to induce apoptosis (cell death) in HL60 cells when analyzed for the presence of DNA fragments [121].

Lupanine was investigated for its mutagenic potential. The study assessed its impact on Salmonella typhimurium strains TA97, TA98, TA100, TA102, TA1535, and TA1538, both with and without the presence of exogenous metabolic activity. The findings indicated that lupanine did not induce mutations in any of these bacterial strains. [122]

Sparteine's potential to induce single-strand breaks was evaluated using the Comet assay, which involved testing on Tradescantia dendritic nuclei. It's important to note that this assay was not standardized. The results of the study showed a mild increase in survival time at a single dose of sparteine. In the same study, ethanol extracts obtained from *L. mexicanus* and *L. montanus* were also examined for their effects on single-strand breaks in the same cells. Interestingly, regardless of the dose or concentration used, these ethanol extracts were found to increase single-strand breaks in the tested cells. This suggests that the extracts from *L. mexicanus* and *L. montanus* had an impact on DNA integrity in this experimental context [123].

### 2.5.7. Neurotoxicity

In a study involving mice, the effects of administering lupanine and sparteine at their highest non-lethal intraperitoneal doses (64 mg/kg for lupanine and 25 mg/kg for sparteine) were investigated, specifically in relation to behavior, psychomotor activity, and exploration. [124]

Both alkaloids were found to induce a slight decrease in spontaneous activity and reduced locomotor activity. These observations suggest that lupanine and sparteine had a mild sedative effect on the central nervous system. [124]

Furthermore, the study explored the interaction of these alkaloids with drugs that affect the central nervous system, as well as their potential analgesic effects. It was determined that lupanine and sparteine did not interact with drugs such as amphetamine and pentetrazol, which stimulate the central nervous system, nor did they interact with pentobarbital and chlorpromazine, which have a depressive effect on the central nervous system. [124]

Additionally, these alkaloids did not demonstrate any analgesic effects in the context of alleviating abdominal cramps induced by acetic acid. Overall, the study's findings indicate that lupanine and sparteine had a sedative effect but did not exhibit interactions with drugs affecting the central nervous system or demonstrate analgesic properties. [124]

In a study conducted on adult Swiss-Wistar rats (with 10 animals per group), the effects of administering QA extracted from the seeds of *L. exaltatus* and *L. montanus* into the intraventricular space of the brain (ICV) were investigated. The rats were subjected to daily ICV injections of sesame oil for 5 consecutive days in the control group, while the AQ extract was administered to the experimental group. [125, 126]

The primary QAs present in the *L. montanus* extract included sparteine (35,000 mg/kg), lupanine (17,700 mg/kg), 3-β-OH-lupanine (3,800 mg/kg), and 13-α-OH-lupanine (3,200 mg/kg). In contrast, the extracts from *L. exaltatus* contained lupanine (5,800 mg/kg), 3-β-OH-lupanine (1,500 mg/kg), and only small amounts of sparteine (284 mg/kg). [125, 126]

Immediately after administering QAs, the rats exhibited various clinical signs, including tachycardia, tachypnea, myoclonus, tail elevation, muscle twitching, lack of balance, euphoria, and an unsteady gait. [125, 126]

At the conclusion of the treatment, the left, uninjured hemisphere of the rats' brains was analyzed. The study revealed a significant number of degenerative changes (approximately 30-40%, compared to around 10% in the control group) in the brains of the AQ-treated animals, particularly in regions such as the thalamus, hypothalamus, and hippocampus. These brain regions are particularly sensitive to QA-induced neurotoxicity because they contain cholinergic neurons and systems involved in the cholinergic pathway, as well as basal ganglia systems with cholinergic receptors (muscarinic or nicotinic types). [125, 126]

The study's findings suggest that QAs induce neuronal damage in the central nervous system. Additionally, it was observed that QA-induced neuronal damage was more severe in *L. montanus* compared to *L. exaltatus,* which is consistent with the higher content of sparteine found in *L. montanus* QA extracts. [125, 126]

When quinolizidine alkaloids (QA) were administered either into the intraventricular space or intraperitoneally in Wistar rats (with 8 animals per group) for a duration of 5 days, the study revealed that certain brain regions in the central nervous system exhibited toxic effects. These regions are commonly associated with cholinergic pathways and include the frontal cortex, prefrontal cortex, striatal and olfactory areas, amygdaloid areas, ventral hypothalamic nucleus, cerebellar Purkinje cells, and gyrus 1 and 3 dentate areas of the hippocampus *Cornus Ammonis.* [127]

In a separate study involving neonatal Wistar rats (again with 8 animals per group), the effects of sparteine on brain damage were investigated. The rats received a single dose of 25 mg/kg body weight via subcutaneous injection on days 1 and 3 postpartum. The observed effects manifested after 7 days and persisted for up to 21 days, and they were characterized by nerve cell death due to necrosis. Importantly, these effects were significantly reduced when the rats were pretreated with atropine, indicating that the cytotoxicity of sparteine is mediated by muscarinic acetylcholine receptors (mAChRs). Additionally, there was a rapid decrease in the expression of m1-m4 mAChRs subunits at both the RNA and protein levels, suggesting an involvement of these receptors in sparteine-induced neurotoxicity. [128]

## 2.6. Free Radicals and Reactive Species

### 2.6.1. Free Radical

Free radicals are highly reactive chemical species characterized by having one or more unpaired electrons in their outermost shell. This unpaired electron makes them chemically unstable and prone to initiating various chemical reactions. There are different types of free radicals, categorized based on the atom or element at the center of the unpaired electron:

1. Oxygen-centered radicals (ROS): These radicals involve oxygen atoms and include species like superoxide (O2•-) and hydroxyl (OH•) radicals. They play a role in oxidative stress and cellular damage. Radical reaction shown in Figure 9 below.

2. Nitrogen-centered radicals (RNS): Nitrogen-centered radicals involve nitrogen atoms and include species such as nitric oxide (NO•) and peroxynitrite (ONOO−). They have both physiological and damaging effects when produced excessively.

3. Carbon-centered radicals (RCOS): Carbon-centered radicals feature an unpaired electron on a carbon atom. An example is the trichloromethyl radical (CCl3•).

4. Sulfur-centered radicals (RSS): These radicals involve unpaired electrons on sulfur atoms. An example is the thiyl radical (RS•).

Free radicals are often represented by a dot (•) next to the atom carrying the unpaired electron(s).

While most biomolecules do not naturally exist as radicals, interactions between radical species and biomolecules can lead to the generation of new free radicals. For instance, when hydrocarbons within the fatty acid side chains of lipids react with hydroxyl radicals (OH•), a hydrogen atom (H•) can be abstracted from the hydrocarbon, resulting in the formation of a carbon-centered radical within the fatty acid chain.

The reactivity of free radicals can have significant consequences in various chemical and biological processes, including oxidative damage to biomolecules such as RNA, DNA, proteins, and lipids. This damage can contribute to various health-related issues, including neurodegenerative diseases, inflammation, atherosclerosis, aging, cardiovascular diseases, and cancer. [129-133, 135, 145]

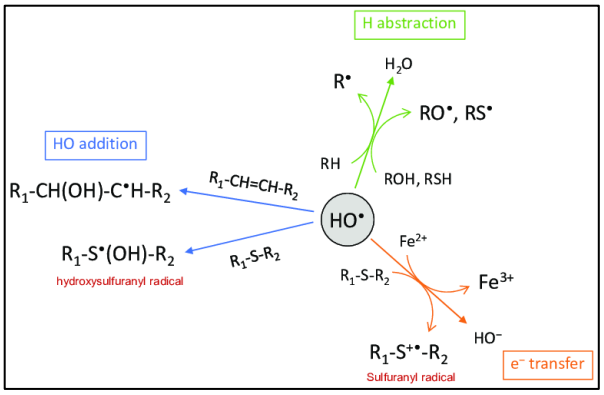



**Figure 9.** An illustration of the Hydroxyl radical reaction. [273]

The reaction depicted in Figure 9 involves the hydroxyl radical (•OH) and is associated with the chain reaction of lipid peroxidation. Which is a process where free radicals attack and damage the lipids (fats) in cell membranes. Here's a breakdown of this process:

5. Formation of Carbon-Centered Radicals: Initially, carbon-centered radicals are generated within the fatty acid side chains of lipids. These radicals have unpaired electrons, making them highly reactive.
6. Reaction with Oxygen: Carbon-centered radicals react with oxygen (O2) to produce peroxyl radicals (ROO•). This reaction involves the addition of oxygen to the carbon-centered radical.
7. Targets for Peroxyl Radicals: Peroxyl radicals (ROO•) are particularly damaging because they can attack adjacent fatty acid side chains in cell membranes and also interact with membrane proteins. These targets are primary sites of peroxidation.
8. Oxidation of Fatty Acid Side Chains: Peroxyl radicals (ROO•) can oxidize the fatty acid side chains of lipids by abstracting hydrogen atoms from neighboring molecules. This results in the formation of lipid hydroperoxides (LOOH).

The chain reaction of lipid peroxidation can propagate further, as lipid hydroperoxides can themselves generate new radicals when they decompose or react with other molecules. Lipid peroxidation is associated with a range of health-related issues, including inflammation, aging, cardiovascular diseases, and neurodegenerative diseases. It is a significant contributor to oxidative stress, which is a condition characterized by an imbalance between the production of free radicals and the body's ability to neutralize them through antioxidant defenses. [135, 136]

### 2.6.2. Reactive Oxygen Species

Reactive Oxygen Species "ROS" refers to a group of molecules that contain oxygen and can be either radicals, such as $O_2$• and OH•, or non-radical derivatives like hydrogen peroxide ($H_2O_2$) and singlet oxygen. These non-radical derivatives have the ability to oxidize biomolecules and/or easily become radicals. Similarly, RNA includes both radical and non-radical reactive molecules, such as $HNO_2$ and $N_2O_3$ [134]. The human body produces free radicals and reactive species as part of important processes like inflammation and neurotransmission, or as byproducts that don't serve a crucial function in the body. During oxidative phosphorylation, the majority of oxygen binds to

hydrogen and is reduced, but a small percentage (1-3%) is not fully reduced and these molecules are collectively known as ROS. Singlet oxygen ($1O_2$), superoxide ($O_2$•-), hydrogen peroxide ($H_2O_2$), hydroxyl radical (OH•), ozone ($O_3$), and hypochlorous acid (HOCl) are the main ROS that are biologically important [135]. Reactive Oxygen Species (ROS) represent a significant class of free radicals with vital roles in living systems. They are produced from molecular oxygen in various physiological processes, particularly redox reactions. The ROS family encompasses both charged species, such as the radical OH•, superoxide (O2•), alkoxyl (RO•), and peroxyl (ROO•), as well as uncharged species, like dioxygen ($1O_2$) and hydrogen peroxide ($H_2O_2$). The generation of ROS within biological systems arises from the reduction of monovalent molecular oxygen in the presence of non-human electrons, leading to the formation of $O_2$•. Any metal-containing molecule present in this mixture can further reduce $H_2O_2$, producing highly reactive compounds [131,143]

The reactivity of ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) varies considerably among them. Certain species, like H2O2 and O2•, NO•, OH•, exhibit selective reactivity towards specific molecules, whereas others, notably OH•, interact with nearly all encountered molecules. Additionally, reactive species have distinct sites within biological systems where they engage in reactions. Generally, free radicals tend to react with their target molecules at the sites of their formation. In contrast, non-radical species like $H_2O_2$ possess the ability to traverse biological membranes, exerting toxic effects at distant locations. [136, 137]

Within cells, various enzymatic pathways are responsible for ROS generation, with superoxide being a prominent example. Superoxide is produced through the one-electron reduction of molecular oxygen and is subsequently converted to hydrogen peroxide by mitochondrial superoxide dismutase. While hydrogen peroxide is less reactive, it plays a pivotal role in generating highly damaging hydroxyl radicals through the Fenton reaction. The electron transport chain, a vital component of oxidative phosphorylation, serves as a major source of superoxide production. As electrons depart from the electron transfer complexes, they reduce oxygen, giving rise to superoxide. Additionally, other mitochondrial reactions can serve as sites for superoxide production, with Cytochrome P450 and α-ketoglutaric acid dehydrogenase being implicated in ROS production. Furthermore, reactions involving nicotinamide adenine dinucleotide

phosphate oxidase (NADPH oxidase), xanthine oxidoreductase and myeloperoxidase are also capable of generating ROS. [138-140]

The presence of ROS (Reactive Oxygen Species) in the human body does not always imply a harmful situation. In fact, ROS serves as an essential component in maintaining redox homeostasis, thereby ensuring the normal functioning of the immune and cardiovascular systems. When ROS levels rise and the body's natural processes cannot effectively manage them, oxidative stress may occur. Conversely, if ROS levels are insufficient, it can lead to a state of reduced stress, which in turn can contribute to various illnesses such as cancer and cardiomyopathy. [138, 141, 142]

ROS plays a pivotal role in specific signaling pathways by interacting with cysteine residues within amino acids and influencing processes related to signal transduction. Proper cell proliferation and apoptosis, for instance, depend on the presence of ROS. Within these processes, ROS-induced cysteine oxidation on enzymes involved in MAPK signaling can protect them from the inhibitory effects of phosphatases. Additionally, ROS can inhibit tyrosine phosphatases through oxidation, thereby helping maintain appropriate levels of growth factors. [138, 141, 142]

Given its critical role in antioxidant mechanisms, ROS products have the capacity to activate antioxidant genes through specific mechanisms like the antioxidant response element (ARE) and the involvement of factors such as PI3K and NFE2 like 2 (Nrf2). The endonuclease Redox factor 1 (Ref1), which is regulated by factors like p53, activating protein 1 (AP1), nuclear factor kappa B (NFκB), and hypoxia-inducible factor 1, moves to the nucleus and initiates an antioxidant defense system when it encounters ROS in the cytoplasm. [138, 141, 142]

Furthermore, ROS plays a vital role in the immune system, particularly in cells associated with innate immunity such as macrophages, dendritic cells, and neutrophils. These cells activate NADPH oxidases during the process of phagocytosis to generate ROS, which is instrumental in destroying foreign entities. [138, 141, 142]

In summary, ROS is generated during metabolic processes and is involved in various physiological functions, including oxidative phosphorylation, enzymatic reactions, activation of nuclear transcription factors, signal transduction, gene expression, and antimicrobial activity within the context of innate immunity. [138, 141, 142]

ROS can originate from both endogenous and exogenous sources. Endogenous sources within mammals comprise cellular organelles, enzymes, and endogenous

chemotactic substances. While mitochondria serve as the primary source of ROS, other cellular organelles, including peroxisomes, neutrophils, macrophages, and neurons, also contribute to ROS production. Enzymes responsible for ROS production in mammals encompass cytochrome P450 enzymes, monoamine oxidase enzymes, xanthine oxidase (XOD)/dehydrogenase (XDH) enzymes, and membrane enzymes like NADPH. Certain substances, such as arachidonic acid (a polyunsaturated fatty acid), catecholamines, prostaglandins, and hemoglobin, are known to trigger ROS formation. [144-147]

ROS play pivotal roles in gene expression, signal transduction, activation of cellular signaling cascades, apoptosis, and function as intracellular and intercellular messengers. Exogenous sources of ROS include ionizers, anesthetic gases, UV radiation, drugs (e.g., bleomycin and adriamycin), chemicals (e.g., alcohol), toxins, pesticides, and xenobiotics (e.g., alcohol). [144-147]

Excessive ROS production is well-documented to instigate various forms of cell death, including necrosis, apoptosis, and autophagic cell death. In the necrotic pathway, the formation of ROS triggers Lipid Peroxidation (LPO), which can subsequently elevate intracellular calcium levels. This, in turn, leads to the opening of the mitochondrial permeability transition pore (MPTP) and compromises cell integrity, resulting in necrosis characterized by cell swelling and/or rupture. Moreover, heightened ROS levels can induce DNA damage by chemically modifying sugars and bases, causing denaturation and depurination. This DNA damage can activate apoptosis through p53 activation, MPTP pore opening, and initiation of the caspase signaling cascade. [132, 148, 149]

Furthermore, the overproduction of ROS can result in the inactivation of Autophagy-Related Gene 4 (ATG4), leading to the accumulation of Autophagy-Related Gene 8 (ATG8), a crucial component required for autophagosome formation. Autophagosomes are round structures enclosed by a bilayer membrane. ROS also have the capacity to oxidize amino acids such as arginine, lysine, and threonine, inducing protein-protein crosslinking and thereby causing damage to protein structures. This damage can disrupt the normal function of enzymes, receptors, or even transport proteins [132, 148, 149]

### 2.6.3. Reactive Nitrogen Species

Reactive Nitrogen Species (RNS) represent a group of free radicals that originate from nitric oxide (NO•) through various physiological processes. The RNS family includes several key species:

9. Nitric oxide (NO•), produced by nitric oxide synthase (NOS).
10. Nitrite (NO2-), generated from NO•.
11. Dinitrogen trioxide (N2O3), produced from NO• and O2.
12. Nitrogen dioxide (NO2•), resulting from the decomposition of peroxynitrite (ONOO-).
13. Nitronium ion cation (NO2+), generated from the decomposition of ONOOCO2-.
14. Nitrosonium ion cation (NO+), produced from NO•.
15. Anion nitroso peroxy carbonate (ONOOCO2-), formed from ONOO- and CO2.
16. Nitroxyl (HNO), produced by the one-electron reduction of NO•.
17. Nitrite chloride (Cl-NO2), generated from NO2 and HOCl.
18. Peroxynitrite (ONOO-), formed from the reaction between NO• and O2•.
19. S-nitrosothiols (RSNOs), resulting from the addition of a NO• group to cysteine thiol/sulfhydryl.

These RNS species play a crucial role in detoxifying $O_2$•-, thereby preventing damage induced by Reactive Oxygen Species (ROS). However, they also play a pivotal role in the oxidation of essential biomolecules such as carbohydrates, proteins, lipids, metal cofactors, DNA, and RNA. [150]

An excessive presence of RNS in living cells can lead to the increased production of sphingolipids like ceramide and its derivatives (e.g., sphingosine), ultimately causing oxidative and nitrosative stress in the cell. This stress results from the activation of NADPH oxidase and NOS, as well as disturbances in mitochondrial function. Nitrosative stress is conceptually analogous to "oxidative stress" and occurs when the production of RNS surpasses the capabilities of the antioxidant defense system. [151]

NO•, $NO_2$•, and ONOO- can cause damage to DNA and RNA through basal and sugar-related mechanisms. This damage can lead to apoptosis, elevated mutation rates, and increased cellular proliferation, all of which are indicators of tumorigenesis. It also functions as an anti-inflammatory agent by inhibiting the production of inflammatory

cytokines, including prostaglandin E2, thromboxane, and interleukin 6. Moreover, NO• suppresses the production of $O_2$• by neutrophils by acting on NADPH oxidase. [152-156]

NO• is rapidly generated in various tissues, and its small size allows it to efficiently penetrate cell membranes and diffuse over distances of several micrometers. NO• plays critical roles in neurotransmission, immune defense, anti-inflammatory responses, cardiovascular system vasodilation, and apoptosis. In the context of apoptosis, NO• has a dual role: high levels of NO• promote apoptosis through ceramide synthesis, while normal levels of NO• inhibit apoptosis by blocking ceramide production. [150, 157, 158]

### 2.6.4. Reactive Carbonyl Species

Reactive Carbonyl Species (RCS) constitute a category of free radicals containing one or more highly reactive carbonyl groups. RCS are notorious for their detrimental impact on various organic molecules, including carbohydrates, proteins, cell membranes, lipids, DNA, and RNA. These species can be generated either endogenously within the body or come from external sources in the surrounding environment. [159]

One notable endogenous pathway for RCS generation is the polyol pathway, which involves a two-step process where glucose is converted to sorbitol through reduction, and sorbitol is then further converted to fructose. RCS can also originate from non-enzymatic processes, including the oxidation of amino acids, lipid peroxidation (LPO), and glycation. Some examples of RCS produced through these processes include acrolein, glycolaldehyde, glyoxal, methylglyoxal, 2-hydroxypropanal, and others. Exogenous sources of RCS encompass factors like smoke, browning agents, additives, vehicle emissions, medications, and various industrial pollutants. [159]

Elevated levels of RCS within living cells can lead to a condition known as "carbonyl stress," characterized by increased oxidation of carbohydrates and lipids or inadequate detoxification of reactive carbonyl compounds derived from both carbohydrates and lipids through oxygen chemistry. Carbonyl stress has been associated with aging, atherosclerosis, diabetes, obesity, kidney failure, and heart disease. Furthermore, RCS play roles in inflammation, abscess formation, and necrosis, contributing to their negative effects. [160,161]

However, it's essential to note that RCS also have beneficial roles when present in low levels. They serve as vital cellular signaling messengers, help maintain metabolic

homeostasis, regulate gene expression, contribute to the immune response, and enable adaptation to various stressors. [162, 163]

### 2.6.5. Reactive Sulfur Species

Reactive Sulfur Species (RSS) refer to molecules containing at least one redox-active sulfur atom or sulfur-containing functional group within their structure. These species have the capability to either oxidize or reduce biomolecules under biological conditions, thereby initiating or influencing significant cellular signals or major biological events. The diverse RSS family includes various sulfur-containing compounds such as elemental sulfur, hydrogen sulfide ($H_2S$), hydrogen disulfide, disulfides, thiols, thiyl radicals, hydro persulfates, polysulfides, hydro polysulfides, iron-sulfur complexes, nitrosothiols, sulfoxides, sulfonic acids, sulfenic acids, sulfonic acids, sulfates, and thiosulfates. [164, 165]

When present in excessive amounts within cells, RSS can oxidize and damage critical biomolecules, leading to oxidative stress. However, at lower concentrations, RSS exhibit beneficial effects. For instance, hydrogen sulfide ($H_2S$) plays a pivotal role in processes like angiogenesis, anti-inflammatory responses and vasodilation. Thiols function as cellular redox buffers, helping to reduce Reactive Oxygen Species (ROS) and thereby maintaining the cellular redox balance. Other advantageous roles of RSS encompass their participation in cellular signaling, the maintenance of redox homeostasis, electron transport within mitochondria, and the regulation of metabolic processes. [166-168]

## 2.7. Damaging Effects of Free Radicals

Free radicals, as *e*ffects on *c*ellular *c*omponents shown Figure 10 below, which are highly reactive chemical species, can damage various biomolecules in cells such as DNA, lipids, proteins, carbohydrates, and fatty acids. When the levels of free radicals become too high, they can overwhelm the cell's natural defense mechanisms and lead to cellular problems. The type of cellular damage caused by oxidative stress can vary depending on the specific cell type, the level of stress, and the extent of exposure to free radicals. For example, oxidative stress caused by hydrogen peroxide ($H_2O_2$) can damage DNA, while stress caused by carbon tetrachloride may result in lipid peroxidation. [112, 113, 127]

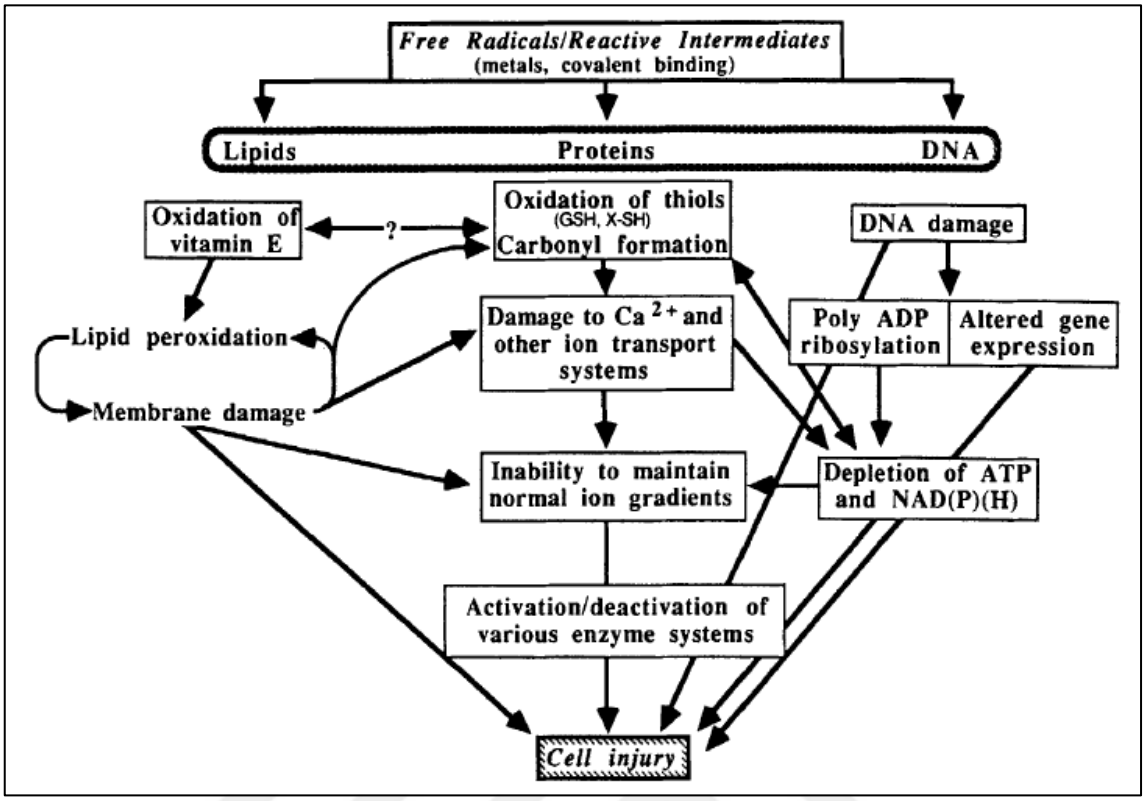



**Figure 10.** Free radicals/reactive intermediates and effects on cellular components [113]

### 2.7.1.1. Effects on Nucleic Acids and DNA

Free radicals are highly reactive molecules that, when present at elevated levels beyond the body's defense mechanisms, can inflict harm on various cellular components including DNA, proteins, carbohydrates, lipids, and fatty acids. This damage has the potential to result in cell death and genetic mutations. For instance, hydrogen peroxide, produced by active neutrophils, can easily permeate cell membranes and enter cell nuclei, where it can harm DNA, leading to dysfunction and cell demise. Hydroxyl radicals also interact with deoxyribose and DNA bases, causing damage. DNA is particularly susceptible to free radical-induced damage. [128-130]

The detrimental effects of free radicals extend to multiple areas, such as chronic inflammation, infections, aging, carcinogenesis, neurodegenerative diseases, and cardiovascular disorders. DNA is especially vulnerable to free radical damage, as these

radicals can induce chain breaks and cleavage in purine and pyrimidine bases. Moreover, free radicals can impact DNA polymerases and DNA repair enzymes, further exacerbating genetic material damage. [130-133]

#### 2.7.1.2. Effects on Proteins

Excessive production of ROS and RNS can result in oxidative stress, leading to the potential damage of DNA, proteins, lipids, and other biomolecules. This, in turn, may contribute to a range of health issues, including inflammation, aging, cancer, and neurodegenerative disorders. Conversely, when ROS and RNS are present at low levels, they serve critical roles in signaling, maintaining redox balance, supporting immune defenses, and facilitating various biological functions. [135-139]

Reactive carbonyl species (RCS), another category of free radicals, can be generated either endogenously or exogenously. Elevated RCS levels can induce carbonyl stress, a condition associated with various diseases such as diabetes, obesity, and heart disease. [135-139]

Sulfur-containing radicals (RSS), yet another class of free radicals, have the capacity to interact with biomolecules and influence their function. While high RSS levels can lead to oxidative stress, lower levels can have beneficial effects, including acting as signaling molecules and participating in redox homeostasis. [135-139]

#### 2.7.1.3. Effects on Carbohydrates

Free radicals can exert a substantial influence on carbohydrates. The autooxidation of monosaccharides can result in the generation of compounds like $H_2O_2$, peroxides, and oxoaldehydes. These compounds can be detrimental to the body and contribute to the onset of diseases such as cancer, diabetes, and chronic conditions associated with smoking. Oxoaldehydes, in particular, possess the capacity to bind to DNA, RNA, and proteins, forming cross-links between these biomolecules. This can induce antimitotic effects and potentially have implications in the fields of oncology and aging. [140]

#### 2.7.1.4. Effects on Membrane Lipids

Free radicals can inflict damage on cell membranes through a mechanism known as lipid peroxidation, the three stages involved in Lipid Peroxidation shown in Figure 11 below. This process involves the oxidation of polyunsaturated fatty acids within the

membrane by free radicals, ultimately resulting in the disruption of the membrane's structural integrity. The stability and flexibility of the cell membrane are crucial for preserving the overall integrity of the cell, and any harm inflicted upon the membrane's lipids can have adverse consequences for the cell. [96]

**Non-Enzymatic Lipid Peroxidation**

Non-enzymatic lipid peroxidation is a complex process characterized by three distinct phases: initiation, propagation, and termination.

1. Initiation: The initiation phase is triggered by the generation of free radicals, which can arise from various sources such as radiation, environmental pollutants, and cellular metabolic processes. These free radicals have the capacity to attack the double bonds found in the unsaturated fatty acids present within the cell membrane's phospholipids. This attack renders these fatty acids unstable.
2. Propagation: During the propagation phase, the unstable fatty acids interact with each other, leading to the formation of lipid hydroperoxides. These hydroperoxides, in turn, engage with other unsaturated fatty acids, setting off a chain reaction that results in the production of additional hydroperoxides.
3. Termination: The propagation phase of lipid peroxidation concludes when the reaction is halted by antioxidant molecules present within the body. Antioxidants, such as Vitamin E and Vitamin C, have the capability to scavenge free radicals and put an end to the chain reaction. However, if the rate at which free radicals are generated surpasses the rate at which antioxidants neutralize them, the lipid peroxidation process persists. This ongoing process culminates in the accumulation of damaged phospholipids within the cell membrane, potentially leading to structural impairment of the membrane, cellular dysfunction, and, ultimately, cell death. [125,141–144]

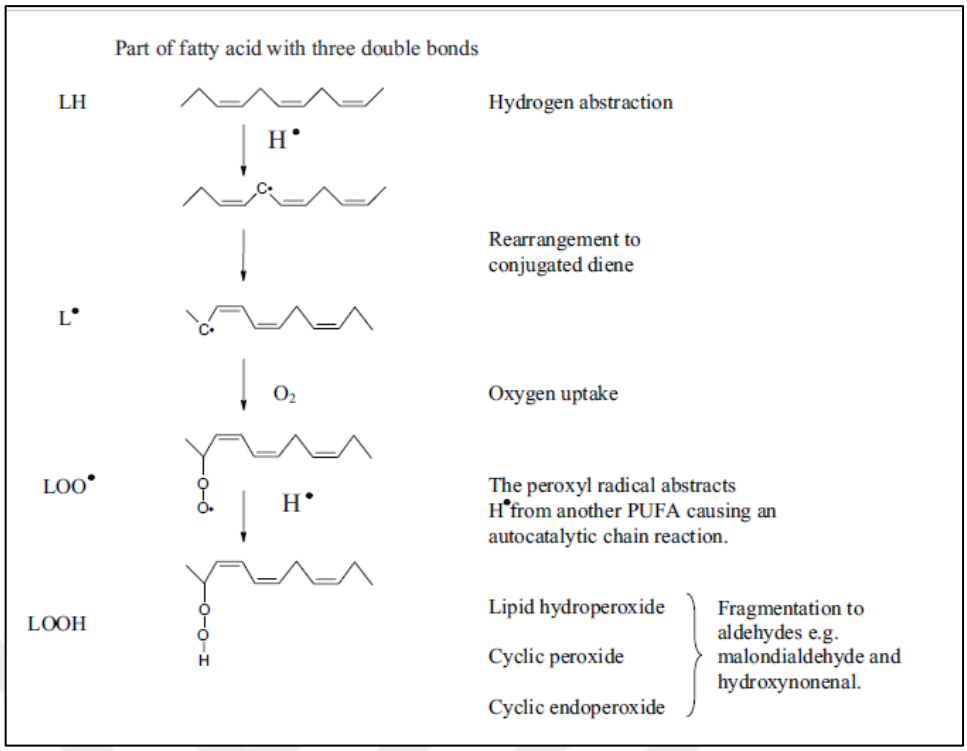



**Figure 11.** Illustrates the three stages involved in Lipid Peroxidation. [125]

The generation of free radicals in an organism initiate when an H+ ion dissociates from a methylene group within a polyunsaturated fatty acid chain located in the cell membrane. The presence of double bonds in the fatty acid weakens the C-H bond, making it more susceptible to detachment. This process can result in the formation of alkoxyl, peroxyl, hydroperoxyl, and OH radicals. The detachment of the H+ atom leads to the formation of a carbon-centered lipid radical within the fatty acid chain. Fatty acids containing one or two double bonds exhibit greater resistance to this form of attack compared to polyunsaturated fatty acids.

During the propagation phase, the lipid radical becomes stabilized by adopting a conjugated diene configuration. Subsequently, the resulting lipid peroxyl radical (ROO•) interacts with molecular oxygen.

The lipid peroxyl radical also engages with adjacent fatty acids, yielding additional lipid radicals (R•). It undergoes a transformation into a lipid hydroperoxide (ROOH) by capturing released hydrogen atoms. This sets off a self-sustaining chain reaction through self-catalysis.

Consequently, numerous fatty acid chains are converted into lipid hydroperoxides. These hydrophilic substances can significantly compromise membrane integrity, fluidity, and permeability. Although lipid hydroperoxides are highly stable, their breakdown can be accelerated by high temperatures and the presence of metal ions, which intensify lipid peroxidation.

The continuation or cessation of these chain reactions depends on factors such as the availability of oxygen and the presence of antioxidants capable of interrupting the chain reaction.

In the final phase of lipid peroxidation, the chain reactions culminate in the collision of two lipid peroxide radicals, resulting in the formation of a non-radical product.

As lipid hydroperoxides decompose, they generate aldehydes and hydrocarbon gases as secondary products. These substances can disperse from the peroxidation site to other areas of the cell, potentially causing damage. [125,141–144]

**Enzymatic Lipid Peroxidation**,

Fatty acids serve as substrates for enzymes such as cyclooxygenase and lipoxygenase, which can induce a degree of lipid peroxidation, leading to the production of hydroperoxides and endoperoxides that possess significant biological activities. Cyclooxygenases, responsible for catalyzing the peroxidation of arachidonic acid, produce prostaglandins [145,146]. The presence of lipid peroxidation can be identified through the detection of MDA, which serves as a marker of peroxidative damage in biological structures. [115]

Polyunsaturated fatty acids, characterized by numerous double bonds like linolenic acid, arachidonic acid, and docosahexaenoic acid, are susceptible to oxidative peroxidation, primarily due to the existence of MDA. Under normal physiological conditions, MDA is a moderately reactive compound with the capacity to act as both an electrophile and a nucleophile. It can engage with proteins containing primary amine groups and interact with physiological components such as nucleic acids and amino phospholipids. In mammals, MDA is swiftly metabolized by aldehyde dehydrogenases, transforming into malonic acid. Malonic acid can inhibit the enzyme mitochondrial succinate dehydrogenase. [96,147]

The MDA test is commonly employed to quantify lipid peroxidation in tissue samples. MDA exists in biological materials in both free and bound forms, with a

relatively small proportion being present as free MDA. Bonded MDA can be released through exposure to strong acid or alkali. [147–149]

## 2.8. Oxidative Stress and Antioxidant Mechanisms

Oxidative stress occurs when there is a disruption in the balance between oxidants and antioxidants, resulting in an excess of oxidants compared to antioxidants. This imbalance can interfere with redox signaling and lead to molecular damage. Oxidative stress can be brought about by either a deficiency in antioxidants or an excessive presence of oxidants. [169]

The human body possesses an antioxidant defense system designed to safeguard cellular components from oxidation caused by free radicals, reactive oxygen species (ROS), and other reactive substances. Antioxidants are substances that can delay or prevent oxidation, even when present in low concentrations. Antioxidants can be categorized into two groups: enzyme-based and non-enzyme-based. Enzyme-based antioxidants, including catalase, superoxide dismutase, and glutathione peroxidase, can interact with reactive species and serve as catalysts before undergoing recycling. Non-enzyme-based antioxidants, such as glutathione, uric acid, and ascorbate, can be either hydrophobic, residing in membranes and lipoproteins, or hydrophilic, located in mitochondria, cells, and nuclear compartments. However, it's important to note that only the reduced form of the antioxidant can carry out this scavenging activity, necessitating the conversion of the oxidized form back into its reduced state. The body maintains a network of antioxidants that mutually reduce each other, ensuring the presence of a reduced form of antioxidants. [170-174]

The non-enzyme antioxidant network, as shown in Figure 12 below, involves substances such as vitamin E, ascorbate, and GSH, which work to counteract lipid peroxyl radicals (LOO•) generated during lipid peroxidation. These antioxidants aid in converting the oxidized state of other antioxidants back into their reduced forms. [137]

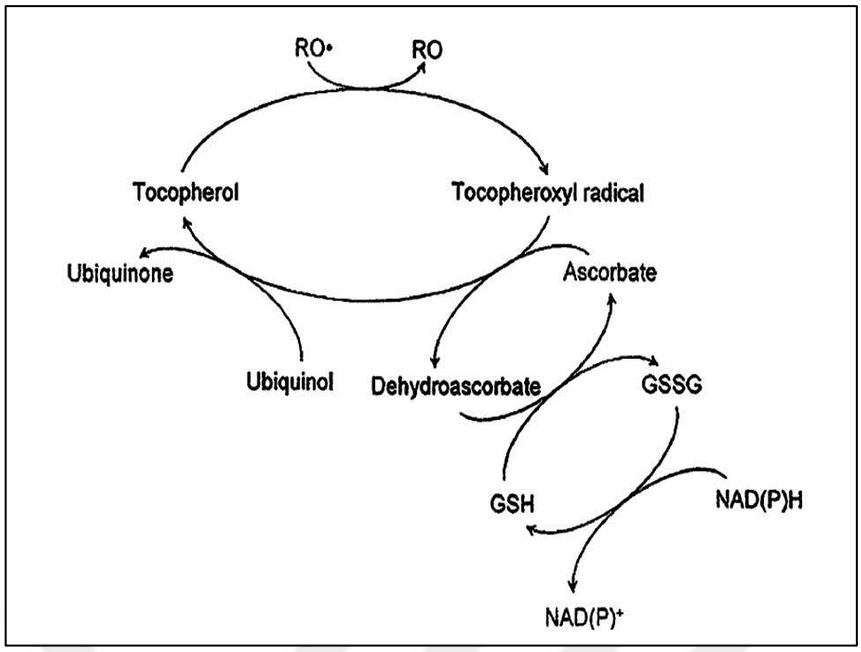



**Figure 12.** Illustrates that the non-Enzyme antioxidant network. [274]

Oxidative stress can arise from external sources like smoking, exposure to pesticides, xenoestrogens, and heavy metals, as well as internal factors such as oxidative bursts triggered by activated macrophages. These factors can elevate intracellular levels of ROS, resulting in oxidative stress and various disorders. Elevated ROS levels can cause structural changes in biomolecules, disrupt the normal operation of mitochondrial membranes, induce mitochondrial dysfunction, and lead to cell death. Additionally, damaged mitochondria can intensify ROS production, notably superoxide anion and hydrogen peroxide, initiating signaling pathways that further amplify ROS generation. [175, 176]

ROS can originate within cells in various locations, including mitochondria, the cytoskeleton, and the extracellular space. Moderate ROS levels play roles in inflammatory responses, activation of mitogen-activated protein kinase (MAPK), and proinflammatory cytokine production. Certain enzymes can also contribute to ROS overproduction, such as myeloperoxidase's generation of hypochlorous acid (HOCl) from hydrogen peroxide (H2O2) during immune responses. Excessive HOCl produced by myeloperoxidases can lead to oxidative stress, apoptosis, and inflammatory diseases. [177, 178]

During the interaction between reactive species and molecules, a hydrogen atom is often extracted, causing the molecules to undergo oxidation. The unpaired electron on the ROS is converted into a more stable electron pair. Alternatively, electron transfer can occur instead of hydrogen atom removal. Due to this effect, reactive species and free radicals are commonly referred to as pro-oxidants. Cell membranes rich in polyunsaturated fatty acids are particularly susceptible to ROS attacks. Reactive species target these fatty acids, removing a hydrogen atom and leaving behind an unpaired electron on the lipid. This results in the formation of a new lipid radical, which seeks to enhance its stability by reacting with oxygen to form a peroxyl radical. The peroxyl radical, in turn, generates a lipid hydroperoxide and another lipid radical by extracting a hydrogen atom from another fatty acid, initiating a chain reaction known as lipid peroxidation. This process can be detrimental to molecular membranes. [134]

Oxidative stress has the potential to induce DNA damage, including oxidized bases, abasic sites, single- and double-strand breaks, which can be mutagenic and result in mismatches during DNA replication. ROS can also lead to protein oxidation, including thiol oxidation and the formation of protein carbonyls. Amino acids containing sulfur, like cysteine and methionine, are particularly susceptible to oxidative attacks by ROS. Oxidation of cysteine leads to the formation of thiyl radicals and disulfides, while ROS reactions with methionine result in methionine sulfoxide formation. These oxidative modifications can disrupt enzyme activity and alter protein binding properties in undesirable ways. [138, 179]

### 2.8.1. Phase I and Phase II Drug Metabolizing Enzymes

Drug metabolism is a vital biological process wherein a living organism readies foreign substances, known as xenobiotics, the xenobiotic reaction shown in Figure 13 below, for either detoxification or activation through specialized enzyme systems. Xenobiotic compounds typically undergo a series of four steps within the body: absorption, distribution, metabolism, and excretion. Upon absorption, a pharmaceutical compound enters metabolic pathways in which it can be detoxified, rendered inactive, or sometimes transformed into potentially toxic active compounds through various enzymatic processes. [180, 181]

Drug metabolism is typically divided into two phases: Phase I and Phase II. In Phase I, enzymes such as cytochrome P450 oxidase facilitate oxidation, hydrolysis, and

reduction reactions. These enzymatic reactions involve the addition of reactive groups, such as hydroxyl radicals, to xenobiotics. The intermediate products formed during Phase I reactions then proceed to undergo further transformation in Phase II. Phase II enzymes carry out conjugation reactions, including processes like glucuronidation, sulphation, acetylation, methylation, and conjugation with molecules like glutathione and amino acids. These Phase II reactions serve to convert xenobiotics into water-soluble compounds, making them easily excretable through urine or bile. [181-183]

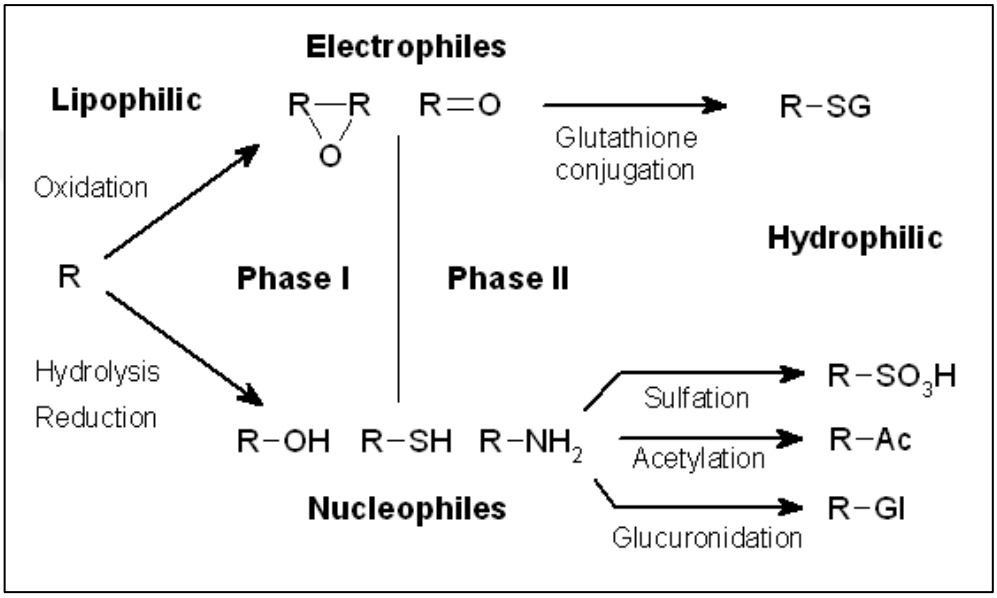



**Figure 13.** Illustrates the process of Xenobiotic metabolism. [275]

#### 2.8.1.1. Phase II Drug Metabolizing Enzymes

Phase II drug metabolism enzymes like Glutathione S-Transferases (GSTs), UDP-glucuronosyltransferases (UGTs), and Sulfotransferases (SULTs) have the capacity to chemically convert active xenobiotics or carcinogens into metabolites that are less toxic or completely inactive. Some enzymes responsible for xenobiotic metabolism are shown in Figure 15 below. [184]

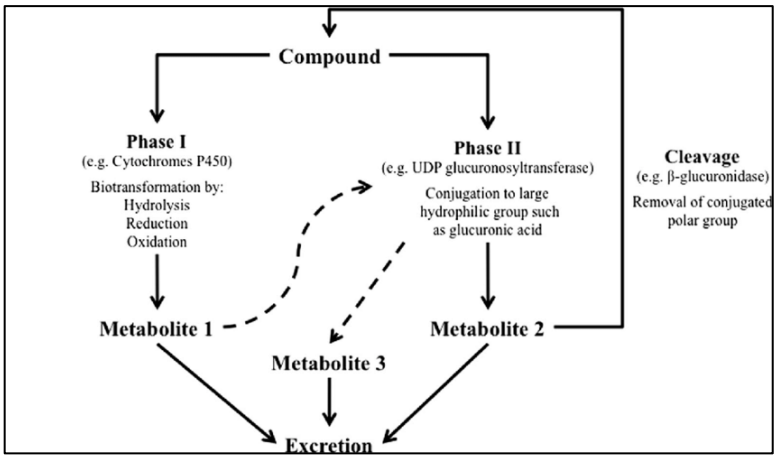



**Figure 14.** Demonstrates inherent differences in the functioning of enzymes responsible for xenobiotic metabolism among various individuals. [276]

Phase II reactions, such as glucuronidation, sulfation, methylation, and amino acid conjugation, can conjugate many xenobiotics into more hydrophilic metabolites for rapid removal from the body. The efficiency of these detoxification reactions can be influenced by various factors including an individual's genetic makeup, the chemical structure of the agent, sex, endocrine status, age, diet, and the presence of other chemicals. [184, 185] Both Phase I and Phase II enzymes can be regulated by phytochemicals through transcriptional regulation or by interacting with enzyme activity. [186,187]

### 2.8.1.2. Glutathione S-Transferases (GSTs)

Glutathione S-Transferases (GSTs), as shown in Figure 15 below, are categorized into three main types: cytosolic, mitochondrial, and microsomal. Among these, cytosolic and mitochondrial GSTs are enzymes that exist in a soluble form, while microsomal GSTs are membrane-bound proteins. The largest group within the GST family is the cytosolic GSTs, which exhibit specific activities. These cytosolic GSTs are further divided into subtypes such as alpha, beta, delta, epsilon, zeta, theta, mu, nu, pi, sigma, tau, phi, and omega, based on the similarities in their amino acid sequences. Glutathione S-Transferases are comprised of N-terminal and C-terminal domains, each featuring a distinct glutathione binding site (GSH) known as the G-site and a hydrophobic site (H-site). [189, 190]

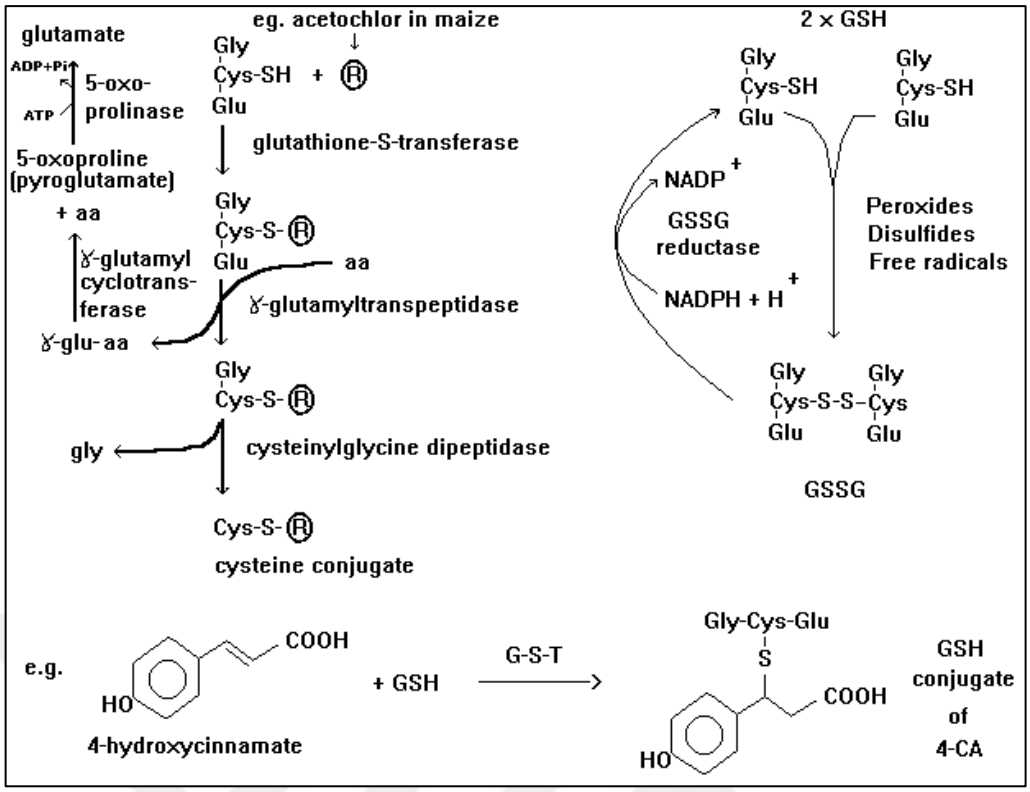



**Figure 15.** Illustrates the presence of Glutathione S-Transferases (GSTs), enzymes that are universally present in all living organisms. [276]

These enzymes are categorized as Phase II detoxification enzymes and play a crucial role in the metabolism of electrophilic substances that contain carbon, nitrogen, and sulfur atoms. They achieve this by combining these substances with the naturally occurring tripeptide glutathione (GSH), resulting in the formation of metabolites that are less reactive and more soluble in water. [188]. The structure of Glutathione *S*-Transferase shown in Figure 16 below.

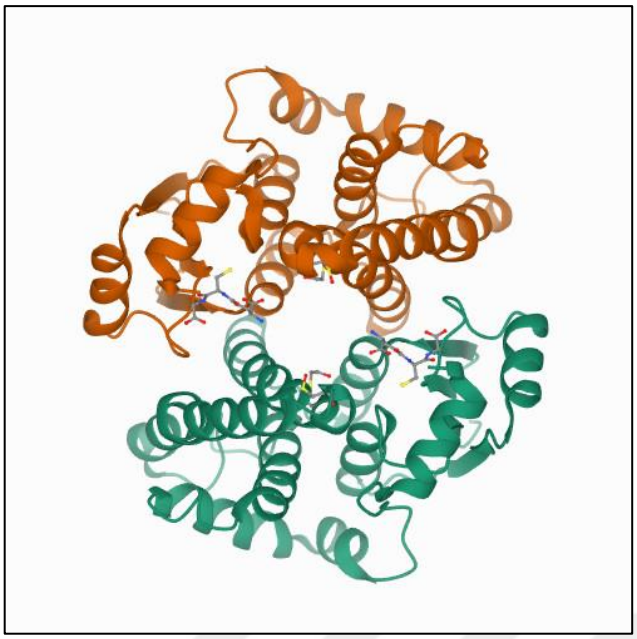


**Figure 16.** Illustrates the crystal structure of human Glutathione S-Transferase (GST) [278]

Glutathione S-Transferases (GSTs) play a crucial role in catalyzing the conversion of prostaglandin H2 into prostaglandin D2, which are mediators involved in allergic and inflammatory responses. The inhibitor nocodazole, which targets GSTS11, is employed as a medication for managing allergies and inflammation. Additionally, certain drugs like cisplatin serves as substrate for GSTP1 and GSTM1 enzymes, respectively, and are eliminated from the body through conjugation with glutathione. [191-193]

GSTs also play a significant role in cancer chemotherapy by metabolizing specific drugs. For instance, the cytotoxic drug TER286 or TLK 286 undergoes metabolism by GSTs to generate the active metabolite cyclophosphamide, a potent medication for treating breast cancer. However, overexpression of GST enzymes can lead to resistance against anticancer agents and carcinogens. As an example, the overexpression of the GSTO1 gene has been observed to confer resistance to cisplatin in HeLa cells. This resistance mechanism reduces apoptosis by activating survival pathways and inhibiting the JNK activation pathway, which is crucial for cellular death. [194-196]

### 2.8.2. Antioxidants

The body possesses a defense mechanism to counteract the harmful effects of free radicals. Antioxidants are substances that can neutralize free radicals and thwart the damage they inflict. These antioxidants can either originate from within the body (endogenous) or be obtained from external sources (exogenous). [197, 198] Tables 1, 2, and 3 outline the various types and roles of antioxidants.

**Table 1.** External antioxidants and their functions. [198]

| **Exogenous Antioxidant** | **Function** |
|---|---|
| Vitamin C (Ascorbic Acid) | Neutralizes free radicals and regenerates vitamin E. |
| Vitamin E (Tocopherol) | Protects cell membranes from oxidative damage. |
| Beta-Carotene | Converts to vitamin A, which is an antioxidant. |
| Selenium | Activates antioxidant enzymes. |
| Flavonoids | Scavenges free radicals and inhibits oxidative enzymes. |
| Zinc | Cofactor for antioxidant enzymes. |
| Copper | Cofactor for antioxidant enzymes. |
| Manganese | Cofactor for antioxidant enzymes. |
| Polyphenols | Neutralizes free radicals and inhibits oxidative enzymes. |
| Coenzyme Q10 | Protects cell membranes and mitochondria from oxidative damage. |
| Lipoic Acid | Regenerates antioxidants like vitamin C and vitamin E. |
| Glutathione | Acts as a cofactor for antioxidant enzymes. |
| Melatonin | Scavenges free radicals and stimulates antioxidant enzymes. |
| Plant Extracts (e.g., Green Tea, Grape Seed) | Contain polyphenols with antioxidant properties. |
| Omega-3 Fatty Acids | Protect cell membranes and reduce inflammation. |
| Astaxanthin | Scavenges free radicals and protects cell membranes. |

These external antioxidants play a vital role in neutralizing free radicals and preventing oxidative damage in the body. [198]

**Table 2.** Internal antioxidants in enzyme structure and their functions [198].

| **Endogenous Antioxidants (Enzyme structure)** | **Functions** |
|---|---|
| Superoxide Dismutase (SOD) | Converts superoxide radicals into hydrogen peroxide and oxygen. |
| Glutathione Peroxidase (GPx) | Reduces hydrogen peroxide to water and lipid hydroperoxides to their corresponding alcohols. |
| Catalase | Decomposes hydrogen peroxide into water and oxygen. |
| Peroxiredoxins (Prx) | Reduces hydrogen peroxide, organic hydroperoxides, and peroxynitrite. |
| Thioredoxin (Trx) | Reduces protein disulfides and maintains protein thiol groups in the reduced state. |
| Glutaredoxin (Grx) | Catalyzes deglutathionylation of proteins. |
| Heme Oxygenase (HO) | Degrades heme into biliverdin, carbon monoxide, and free iron. |
| Biliverdin Reductase (BVR) | Converts biliverdin into bilirubin. |
| Paraoxonase (PON) | Hydrolyzes lipid peroxides and reduces LDL oxidation. |
| NAD(P)H:Quinone Oxidoreductase 1 (NQO1) | Reduces quinones and prevents redox cycling. |
| Metallothionein (MT) | Binds to metal ions and acts as a scavenger of hydroxyl radicals. |
| Ferritin | Stores and releases iron in a controlled manner. |

These endogenous antioxidants, found within the body, play crucial roles in neutralizing harmful reactive species and maintaining redox balance. [198]

**Table 3.** Non-Enzyme Endogenous antioxidants and their functions. [198]

| **Endogenous Antioxidants (Non-enzyme)** | **Functions** |
|---|---|
| Albumin | Scavenges hypochlorous acid radicals. Binds protein and metal ions. |
| Ceruloplasmin | Binds copper ions and participates in the oxidation of copper using H2O2. |
| Transferrin | Inhibits the Fenton reaction by binding free iron ions. |
| Lactoferrin | Binds iron ions in low pH environments. |
| Haptoglobin | Binds hemoglobin. |
| Hemopexin | Binds heme groups. |
| Bilirubin | Acts as a peroxyl radical scavenger. |
| Glucose | Functions as a hydroxyl radical scavenger. |
| Urate | Captures radicals and binds metals. |
| Melatonin | Exhibits antioxidant effects by neutralizing hydroxyl and superoxide radicals. |

These non-enzyme endogenous antioxidants play essential roles in combating oxidative stress and maintaining cellular health. [198]

### 2.8.2.1. Antioxidant Enzymes

Oxidation reactions are characterized by the transfer of electrons from one molecule to another. During this electron transfer process, free radicals are frequently generated. These free radicals have the potential to initiate chain reactions, leading to additional damage. [199]

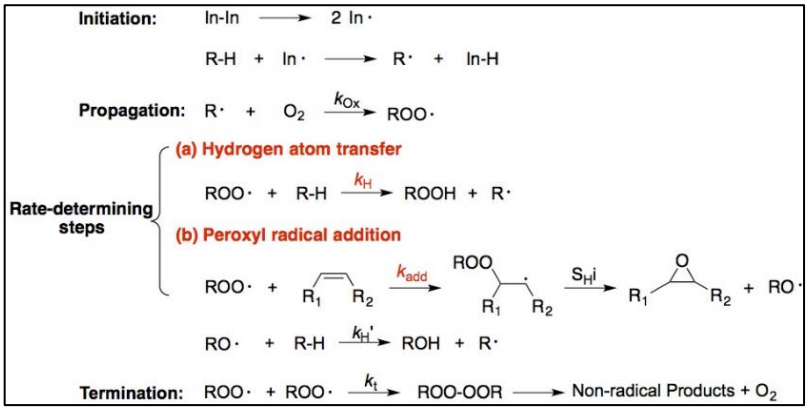



**Figure 17.** Shows free radical lipid Peroxidation. [279]

The body's inherent defense system against oxidative stress comprises both enzymatic and non-enzymatic antioxidants. Enzymes like catalase, superoxide dismutase, and glutathione peroxidase have the ability to engage with reactive species, serving as catalysts and being efficiently regenerated in the process. Non-enzymatic antioxidants, such as glutathione, uric acid, and ascorbate, are primarily hydrophilic and are predominantly located within mitochondria, cells, and nuclear compartments. These non-enzymatic antioxidants act as scavengers for radicals, contributing an electron or proton to reactive species to mitigate their reactivity while undergoing oxidation themselves. The body maintains a network of antioxidants that mutually regenerate and uphold an active reduced state of antioxidants. [199, 200]

**Superoxide Dismutase**

Superoxide dismutase (SOD) is an enzyme responsible for catalyzing the conversion of superoxide, a highly reactive oxygen species, into hydrogen peroxide and oxygen. [231] This crucial process serves to safeguard cells against oxidative damage instigated by superoxide. There exist three isoforms of SOD, each associated with a distinct metal cofactor: Cu/Zn SOD, which is localized in the cytosol; Mn SOD, situated within mitochondria; and Fe SOD, found in peroxisomes. [232] SOD plays a pivotal role in the cellular antioxidant defense system, and its deficiency can give rise to a range of diseases, including cardiovascular ailments and neurodegenerative disorders. [233, 234]

The SOD-mediated process involves electron transfer, leading to the conversion of Cu2+ to Cu1+ and the generation of atomic oxygen. [235] Simultaneously, another superoxide anion captures protons and electrons from the surrounding environment, culminating in the production of hydrogen peroxide, thus concluding the dismutation reaction. [236] There are four primary categories of superoxide dismutase enzymes categorized by the type of metal ion they contain: mononuclear Fe, Mn, and Ni, or dinuclear Cu/Zn. [235] These enzymes can be encountered in the cytosol of eukaryotic cells, the intermembrane space of mitochondria and nuclear membranes, and in some prokaryotes. [236] Both prokaryotes and eukaryotes possess tetrameric mitochondrial dismutase, although the prokaryotic version exhibits a larger molecular weight. Ni-containing SOD is typically present in bacterial strains like Streptomyces, while Fe-SOD is found in various microorganisms, plants, and some anaerobic environments. [231, 237]

**Glutathione Peroxidase (GPx)**

Glutathione peroxidase, as the structure shown in Figure 18 below, is an enzyme that is present in both the cytosolic and mitochondrial compartments of cells. It is characterized by its homotetrameric structure, consisting of four identical subunits, and it is water-soluble. [204]

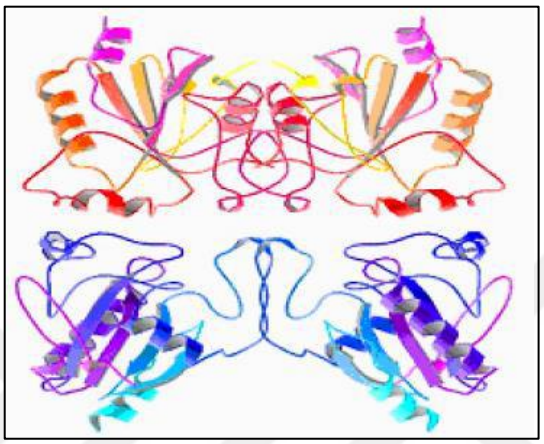


**Figure 18.** Illustrates structure of Glutathione Peroxidase [204]

Glutathione peroxidase (GPx) is an enzyme that plays a crucial role in protecting the body from oxidative damage. It achieves this by reducing hydrogen peroxide to water and alcohol through the utilization of glutathione (GSH). GPx is found in four different isoforms, namely GPx1, GPx2, GPx3, and GPx4, and these isoforms contain selenium as a vital component, making them selenoproteins. [205-209] The Glutathione Redox System shown in Figure 19 below.

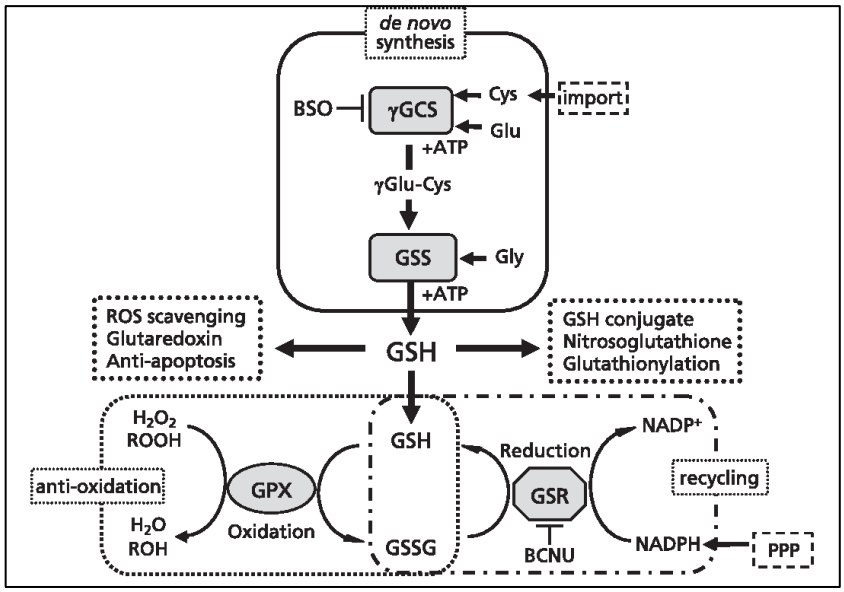



**Figure 19.** Illustrates the Glutathione redox system [280]

Glutathione peroxidase (GSH-Px) was initially identified in animal tissues by Mills. While it is typically absent in plants and microorganisms, it has been detected in certain algae and fungi. [238,239] There are two primary variants of glutathione peroxidase (GSH-Px). One of them is "selenium-dependent GSH-Px (Se-GSH-Px)," which incorporates selenium as selenocysteine into its active site. This type of GSH-Px is capable of efficiently breaking down both $H_2O_2$ and organic hydroperoxides through the oxidation of GSH. Another enzyme known as GSH-Rd is responsible for reducing oxidized glutathione (GSSG) back to its reduced form, GSH. [238,240,241]

The second type of glutathione peroxidase (GSH-Px) exhibits minimal activity against $H_2O_2$ and consists of non-selenium dependent proteins that assist in catalysis. A family of enzymes known as GSTs (Glutathione S-Transferases) plays a crucial role in conjugating GSH with electrophilic chemicals [238].

Selenium-dependent GSH-Px (Se-GSH-Px) consists of four protein subunits, each containing a selenium atom in its active site. The catalytic cycle of Se-GSH-Px is shown in Figure 20 below. This enzyme has an approximate molecular weight of 85,000 and is present in both the cytosol and mitochondria. In the presence of selenocysteine, selenium replaces the sulfur atom in a cysteine amino acid. Se-GSH-Px utilizes GSH to reduce the selenium within the enzyme, which then reacts with $H_2O_2$. [242]

The catalytic cycle of Se-GSH-Px is illustrated in figure 20. During this cycle, the peroxide substrate (ROOH) is converted into alcohol by the selenolate derivative of Se-GSH-Px, which is also transformed into an oxyacid. GSH is introduced into the process, resulting in the formation of selenosulfide. This compound then binds to a second GSH molecule through enzymatic action, converting it into the activated form selenolate and oxidizing the first GSH to GSSG. [238] Se-GSH-Px exhibits the highest activity in the liver, followed by the heart, lungs, and brain, with the lowest activity observed in muscle tissue. It can be found in both the mitochondria and cytoplasm of liver cells. [242]

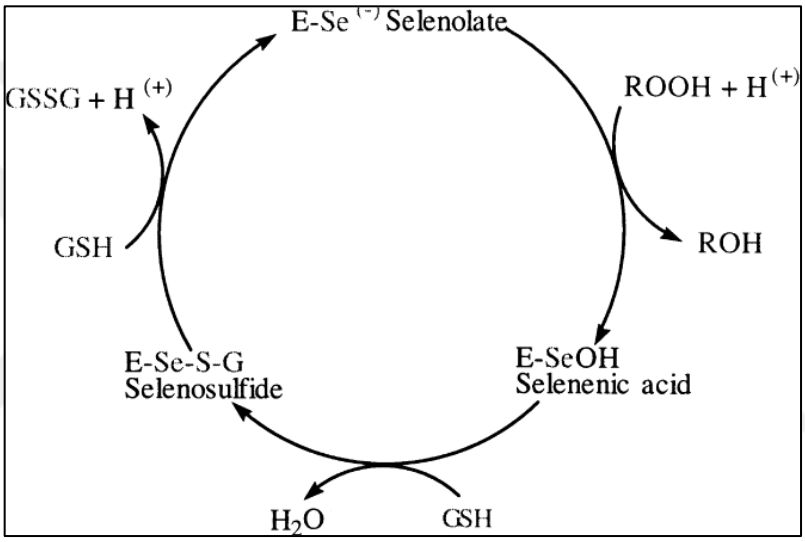



**Figure 20.** Shows the catalytic cycle of Se-GSH-Px [243]

Phospholipid hydroperoxide GSH-Px is another type of GSH-Px enzyme with a molecular weight of 20,000. This cytosolic enzyme contains a single selenium atom and is responsible for converting hydroperoxides of transmembrane lipids into alcohols. Vitamin E, an antioxidant crucial for guarding against membrane oxidation, is particularly effective in cases of deficiency [238].

**Glutathione Reductase (GSH-Rd)**

In the process of reducing $H_2O_2$ or peroxides, GSH-Px converts GSH into GSSG, an oxidized form. To make GSH usable again in this process, the enzyme GSH-Rd comes into play, requiring NADPH for its function. This step is crucial because the organism's

supply of GSH is limited, and recycling it ensures its continued availability. [238,242,244-246]

The primary enzyme system responsible for generating nicotinamide adenine dinucleotide phosphate (NADPH), needed for the reduction of GSSG to GSH by the GSH-Rd enzyme, is the oxidative pentose phosphate pathway. The initial enzyme in this pathway is known as glucose-6-phosphate dehydrogenase (G6PD).

The enzyme 6-phosphogluconate dehydrogenase plays a role in converting 6-phosphogluconate into NADPH, which is essential for the reduction of GSSG to GSH by the GSH-Rd enzyme.

The GSH-Rd enzyme consists of two protein subunits, and each of these subunits contains a FAD molecule at its active center. This enzyme has a molecular mass of approximately 120,000. It is believed that NADPH reduces FAD, leading to the likely breakage of the disulfide bond in GSSG. The resulting electrons are then transferred to the disulfide (-S-S-) bridge, ultimately converting the molecule to GSH. [242]

#### 2.8.2.2. Non-Enzymatic Antioxidants

**Catalase**

Catalase is a protein composed of four subunits, and each of these subunits contains a heme group. This enzyme has a molecular weight of 240 kD. [200] The structure of Catalase is shown in Figure 21 below.

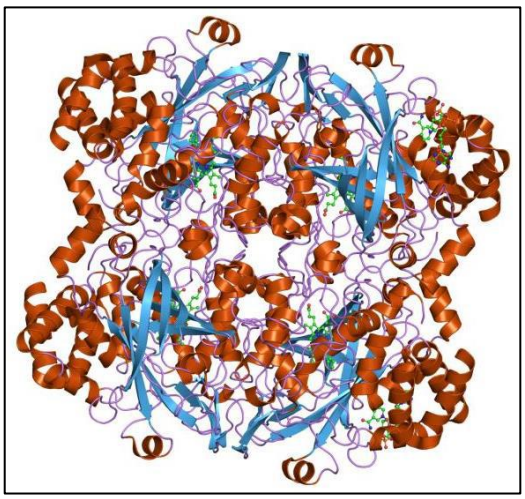


**Figure 21.** Indicates the crystal shape of Catalase [201]

Catalase stands as a highly prevalent enzyme encountered across the majority of living organisms. Its primary function involves facilitating the decomposition of hydrogen peroxide into water and oxygen molecules through catalytic action. [200]

Beyond its role in decomposing hydrogen peroxide, catalase also serves a peroxidative function wherein it transforms peroxides (ROOH) into alcohols (ROH) and water. This enzymatic action serves as a protective mechanism for cells, shielding them from the detrimental effects of lipid peroxidation. Catalase is distributed within various cellular compartments, including peroxisomes, the cytoplasm of human neutrophils, and the cardiac mitochondria of mice. In erythrocytes, which contain substantial amounts of non-peroxisomal catalase, the degradation of hydrogen peroxide is primarily accomplished by GPx. [202, 203]

Catalase (CAT) was initially isolated from bovine liver in 1937 by Sumner and Dounce. Its structural composition consists of approximately 240,000 molecules, with each molecule comprising four protein subunits bound together by an iron group known as Fez-protoporphyrin, which forms the enzyme's active site. Furthermore, each subunit incorporates a reduced NADPH molecule, serving as a protective mechanism against the enzyme's own source of $H_2O_2$ [238, 242, 247].

It's worth noting that the enzyme's activity can be compromised through subunit dissociation, which can occur due to factors like storage conditions, freeze-drying, and exposure to acidic or alkaline environments. Catalase is ubiquitously distributed across the various organs of animals, with its highest concentrations typically found in the liver and red blood cells. It is also present in cellular organelles such as mitochondria, chloroplasts, and the endoplasmic reticulum, with a notable presence in peroxisomes. [248]

Catalase employs a protective mechanism akin to the reaction observed in superoxide dismutase (SOD) to mitigate the detrimental effects of $H_2O_2$. Through this process, catalase effectively converts $H_2O_2$ into oxygen ($O_2$) and water ($H_2O$). [238, 249, 250]

Catalase exhibits the potential to execute certain peroxidase-like activities. In the enzyme's presence, compound I has the capacity to transform aldehydes, such as acetaldehyde and formaldehyde, derived from alcohols like methanol and ethanol. [242]

Under certain in vitro conditions, catalase may be able to oxidize elemental mercury to mercury II. It has also been suggested that catalase can convert nitrite into nitrate. [250]

**Glutathione**

The synthesis of a low molecular weight tripeptide known as Glutathione (GSH) involves the combination of three amino acids: L-glutamic acid, L-cysteine, and glycine. The chemical formula of GSH shown in Figure 22 below. These biochemical reactions are facilitated by both glutathione synthetase and glutamyl cysteine synthetase, and they require the expenditure of one mole of ATP per step. GSH is notably abundant in the liver and is primarily distributed within the cytoplasm, mitochondria, and nucleus of cells. It's important to note that the majority of intracellular glutathione exists in its reduced form (GSH), with the oxidized form (GSSG) being comparatively rare. [238, 246, 251-256]

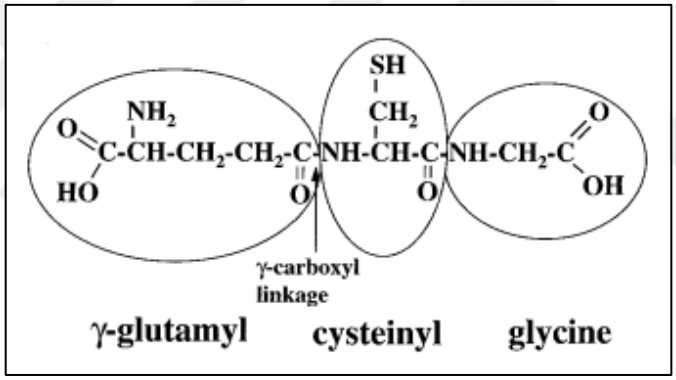



**Figure 22.** Shows the chemical formula for GSH [254]

GSH, owing to its high nucleophilic properties, plays a vital role in the detoxification of electrophilic substances through a process known as conjugation. This process involves the synthesis of thioether compounds, including derivatives of mercapturic acid and N-acetylcysteine, with the resulting byproducts subsequently being excreted in urine. The initiation of this detoxification pathway relies on the catalytic action of the GST enzyme, which facilitates the initial step by donating electrons. [238, 253]

GSH serves as a substrate for various enzymes, including GSH-Px and dehydroascorbate reductase. It also possesses the ability to scavenge hydroxyl radicals (OH•) and singlet oxygen. Additionally, GSH can reduce a wide range of oxidizing agents and reactive species. GSH-Px, in particular, specializes in reducing peroxides, while GSH is involved in the non-enzymatic reduction of free radicals. As previously mentioned, the oxidation of GSH is associated with the breakdown of hydroperoxides and hydrogen peroxide ($H_2O_2$) through the action of GSH-Px. In the presence of NADPH, GSH can be regenerated, and GSH-Rd is responsible for reducing oxidized GSH (GSSG) back to its active form. [238, 242] The cycle of GSH and GSSG shown in Figure 23 below.

Quantification of glutathione levels can be conducted in both blood and tissue using the Ellman method, which entails measuring the absorbance of a colorant generated through the interaction of GSH with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) at a wavelength of 412 nm. It's worth noting that this technique has undergone various modifications and refinements over time. [256, 257, 258]

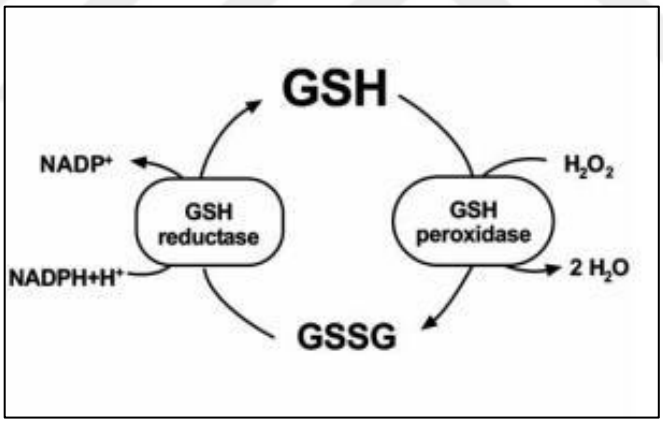



**Figure 23.** Illustrates the GSH and GSSG cycle. [243]

The producing Mercapturic Acid through GSH conjugation is shown in Figure 24 below.

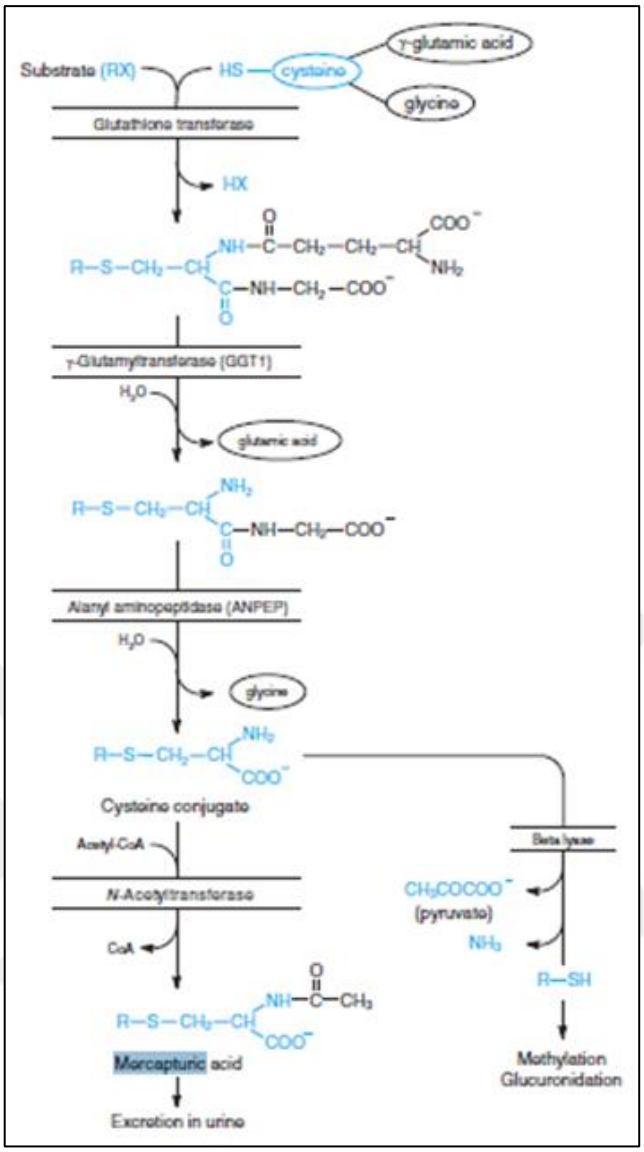



**Figure 24.** Presents the process of producing Mercapturic Acid through GSH conjugation. [255]

### Ascorbic Acid (Vitamin C)

Vitamin C serves as a potent reducing agent in various hydroxylation reactions within the body. It is, however, highly susceptible to oxidation by organic substances. Vitamin C is renowned for its robust antioxidant properties, enabling it to swiftly react with oxygen ($O_2$) and hydroxyl radicals (OH•) to neutralize them effectively.

Additionally, it has demonstrated its ability to prevent oxidative degradation in foods containing oils, such as fish, margarine, milk, vegetable oils, and animal fats. [259]

Nonetheless, it's important to acknowledge that vitamin C's generation of radicals can also result in the production of hydrogen peroxide ($H_2O_2$) and ferrous ions ($Fe^{2+}$), which are essential components of the Fenton reaction. The efficacy of vitamin C as an antioxidant hinge on the ratio of $Fe^{3+}$ to $Fe^{2+}$, and the availability of iron plays a crucial role in determining whether vitamin C functions as a prooxidant or antioxidant. [238, 260]

**α-Tocopherol**

α-tocopherol, the sole fat-soluble antioxidant, is located within plasma lipoproteins, particularly low-density lipoproteins (LDL), as well as within the phospholipid membrane of cell membranes. It possesses a remarkable propensity for readily donating its hydrogen atom from the hydroxyl (OH•) group of tocopherols. This attribute makes it more inclined to associate with nearby fatty acids rather than engaging with peroxyl and alkoxyl radicals that emerge during lipid peroxidation. Consequently, these radicals are effectively intercepted and quenched by α-tocopherol, which plays a crucial role in protecting against lipid peroxidation. [261]

**Carotenoids**

The chloroplast membrane in plants contains β-carotene, which has the capacity to be converted into vitamin A within the body. β-carotene is a highly effective scavenger of singlet oxygen and also exhibits the ability to impede the chain reactions that lead to lipid peroxidation by directly interacting with hydroxyl (OH•), peroxy, and alkoxy radicals. In contrast, tocopherols, although present in lower concentrations, have limitations in protecting against hydrophobic substances due to their solubility. Conversely, lycopene, recognized as the most potent carotenoid, serves as a robust deterrent against singlet oxygen. [238, 262]

**Deferoxamine**

Deferoxamine is a specialized chelating agent with a high affinity for iron, particularly $Fe^{3+}$, making it resistant to reduction. While it reacts relatively slowly with peroxides and oxygen, it demonstrates effectiveness in preventing lipid peroxidation in various systems. Deferoxamine serves to inhibit the generation of hydroxyl radicals (OH•) via iron-dependent hydrogen peroxide ($H_2O_2$) reactions and, simultaneously,

enhances the oxidation of solutions containing $Fe^{2+}$. Additionally, it reacts with newly formed $Fe^{3+}$ ions and efficiently eliminates peroxyl radicals from the system. [242]

**Lipoic Acid**

The conversion of dihydrolipoic acid into lipoic acid results in the production of a compound that possesses the capability to counteract reactive oxygen species and chelate metal ions. Lipoic acid also plays a crucial role in the regeneration of vitamins C and E. [263]

**Melatonin**

Melatonin stands out as a remarkably potent antioxidant primarily because of its lipophilic properties. This quality enables melatonin to effectively scavenge hydroxyl radicals (OH•), making it a versatile and broad-spectrum antioxidant that can operate across various cellular compartments, including cell nuclei, and even penetrate the blood-brain barrier. Importantly, melatonin is distinct in that it does not exhibit prooxidant effects, setting it apart from certain other antioxidants. [254, 265]

**Metallothionein**

Metallothioneins are compact proteins characterized by their cysteine-rich composition, and they have a unique ability to bind to transition metal ions. Additionally, metallothionein serve to impede the News-Weiss and Fenton reactions, processes that produce hydroxyl radicals (OH•), by effectively sequestering and metabolizing metals. [266] Furthermore, metallothionein are associated with the antioxidant properties of melanin, albumin, cysteine, and bilirubin. [238]

**Uric Acid**

In human tissues, urate oxidase is absent, leading to the accumulation of uric acid, a metabolic byproduct of purine metabolism, within the body. Uric acid is recognized as an in vivo antioxidant, and it exhibits remarkable efficacy in scavenging various reactive oxygen species, including singlet oxygen ($O_2$), peroxyl radicals, hydroxyl radicals (OH•), ozone, and hypochlorous acid (HOCl). [267]

When urate interacts with hydroxyl radicals (OH•), it gives rise to the formation of carbon-centered radicals, which subsequently react with oxygen to produce urate peroxyl radicals. While these urate peroxyl radicals are less reactive than hydroxyl radicals (OH•), it's important to note that they should not be regarded as completely harmless. [238]

**Ubiquinol**

Within the mitochondria, the electron transport chain depends on ubiquinol (Coenzyme Q) to facilitate the transfer of protons between the two sides of the inner membrane. Ubiquinol is highly efficient when operating within the lipid phase, and during its two-step oxidation process, it generates superoxide radicals ($O_2$•) while concurrently neutralizing free radicals. [268]

## 2.9. Measurement Methods of Oxidative Stress Parameters

Oxidative stress can be identified through several markers, which include:

1. Malondialdehyde (MDA): A frequently observed product of lipid peroxidation.
2. 8-hydroxy-2'-deoxyguanosine (8OHdG): A marker indicating damage to oxidized DNA.
3. Various antioxidant enzymes such as:
    a. Superoxide dismutase
    b. Glutathione peroxidase
    c. Catalase
    d. Glutathione S-transferase
    e. Glutathione reductase

Additionally, various antioxidant molecules and compounds can serve as markers of oxidative stress, including:

4. Alpha-tocopherol (Vitamin E)
5. Ascorbic acid (Vitamin C)
6. Glutathione
7. Ubiquinone (Coenzyme Q)
8. Cysteine

The presence or levels of these markers can provide insights into the extent of oxidative stress within biological systems [210]. Some of the measurement types shown in Table 4 below.

**Table 4.** Measuring radicals and measurement Types. [210]

| **Measurement** | **Electron paramagnetic resonance spectrometry (EPR)** |
|---|---|
| Measurement of oxidative damage biomarkers | Determination of lipid peroxidation products |
| | Determination of protein damage |
| | Detection of DNA damage |
| Measuring antioxidant defense system | Evaluation of antioxidant enzymes:<br>Superoxide dismutase (SOD)<br>Glutathione peroxidase (GPx)<br>Catalase (CAT)<br>Glutathione-S-transferase (GST)<br>Glutathione reductase (GR) |
| | Determination of total antioxidant activity |
| | Measurement of low molecular weight antioxidants (LMWA); Alpha-tocopherol, Ascorbic acid, Glutathione and Melatonin |
| Measurement of enzyme cofactors | Fe, Cu, Zn, Se, Mn elements |

Numerous techniques are available for assessing oxidative stress within the body. One approach involves the direct quantification of reactive oxygen species (ROS) and reactive nitrogen species (RNS). This can be achieved through methods like fluorescent staining using DCFDA or dihydroethidium, enabling the direct measurement of these species. Another strategy entails evaluating oxidative damage to biological molecules, such as hydroperoxides, utilizing assessments like the derivative of reactive oxygen metabolites test. [211-214]

Indirect methods for gauging oxidative stress encompass the analysis of antioxidant levels within the body and the calculation of specific ratios, such as the ROS/RNS ratio or the ROS/$O_2$• ratio. These approaches provide valuable insights into the assessment of oxidative stress within living systems. [211-214]

Another way to measure oxidative stress is to quantify the amount of damage to RNA/DNA, lipids (LPO), and proteins caused by ROS, RNS, RCS, and RSS. Techniques such as advanced protein oxidation assays and DNPH assays can be used to assess this damage, which is referred to as "protein oxidation" because it results from direct reactions

with ROS and RNS or indirect reactions with secondary oxidative stress. This type of measurement is a useful way to indirectly assess oxidative stress. [215-217]. One way to measure oxidative stress caused by damage to lipids is to measure the levels of malondialdehyde (MDA) using the TBARS assay. MDA is a common byproduct of the chemical reaction between polyunsaturated fatty acids (PUFAs) and ROS. Other methods for measuring oxidative damage to cellular lipids include measuring 8-isoprostaglandin F2α using UHPLC, 4-hydroxy-2-nonenal (4HNE) using HPLC, and levels of oxygenated LDL using the Sandwich ELISA assay and lipid hydroperoxides (LOOH) using the oxidized xylenol orange test. LPO refers to the breakdown of lipids by oxidants such as ROS and RNS. [211, 218]

Measuring the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in biological samples is a way to evaluate oxidative damage to DNA. 8-OHdG is a product of the addition of an OH• group to a deoxyguanosine residue. Its levels can be measured using techniques such as ELISA, HPLC with an electrochemical detector, IHC, and the oxy DNA-FITC conjugated fluorescence assay. Another biomarker of oxidative DNA damage is the accumulation of thymidine glycol (TG) in tissues, which can be assessed by IHC using the streptavidin-biotin-peroxidase complex method. The comet assay is a test that can be used to evaluate oxidative stress in DNA by identifying DNA damage in the form of single- or double-strand breaks. Levels of DNA repair enzymes like human-8-oxoguanine-DNA-glycosylase and apurinic endonuclease can also be measured using IHC or HPLC to estimate the amount of oxidized DNA damage in biological samples. [211, 219, 220- 222] Assessing the levels of antioxidants in the body is another indirect method of measuring oxidative stress in living cells. Enzymes like CAT, SOD, GPx, and GST play a significant role in regulating oxidative stress by controlling the levels of ROS in the body. CAT converts $H_2O_2$ into water and oxygen, SOD converts O2• to $H_2O_2$ and $O_2$, GPx reduces $H_2O_2$ and lipid peroxides to $H_2O$ and their respective lipid alcohols through the chemical oxidation of GSH to GSSG, and GST quenches ROS by adding GSH and protecting cells from oxidative damage. [223-225]

Non-enzymatic antioxidants like glutathione (GSH) and vitamins A, C, and E also contribute to protection by reducing oxidative damage through the removal or neutralization of species generated by free radicals. Therefore, measuring their levels is crucial for assessing oxidative stress. GSH levels can be quantified using methods such as the Ellman reagent, the 5,5'-dithiobis-(2-nitrobenzoic acid) method, GSH-400, and the

2,3-naphthalenedicarboxaldehyde fluorometric assay. Vitamins A, C, and E can be determined using reverse-phase HPLC. [211, 226, 227]

In the process of evaluating the antioxidant activity in a living organism, a specific concentration of the substance being tested is typically introduced into the test subjects. After a period of time, the subjects are usually euthanized and samples of blood or tissue are collected for analysis. [229, 230].

The in vitro methods are as follows [228]:

- DPPH• scavenging activity
- OH• scavenging activity
- OH• averting capacity (HORAC)
- $O_2$• scavenging activity
- NO• scavenging activity
- Peroxynitrite radical scavenging activity
- Total radical-trapping antioxidant parameter (TRAP)
- Biological antioxidant potential (BAP)
- Cupric ion reducing antioxidant capacity (CUPRAC)
- Oxygen radical absorbance capacity (ORAC)
- Ferrous ion chelating activity
- Ferric ion reducing power method
- FRAP assay
- $H_2O_2$ assay
- Ferric thiocyanate (FTC) method
- N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD) method
- β-carotene linoleic acid method/conjugated diene assay.

The in vivo methods are as follows [229, 230]:

- CAT test,
- The ferric reducing ability of plasma,
- GPx estimation,
- GR test,
- GST estimation,
- γ-GGT assay,
- LPO assay,

- LDL estimation,
- GSH estimation,
- SOD estimation.

# 3. MATERIAL and METHODS

## 3.1. Material

The utilization of laboratory animals in this study underwent a thorough ethical evaluation and received approval from the Yeditepe University Experimental Animal Ethics Committee, documented under Decision Number 14/10/2021 - 2021/10-3. For this research, a cohort of 20 adult male and 20 adult female Sprague Dawley rats was sourced from the Yeditepe University Medical Research Center. These animals were housed under standard conditions, which included a 12-hour light/dark cycle and unrestricted access to regular food and water. Their living environment consisted of well-maintained and clean cages.

### 3.1.1. Plant Material

*Lupinus albus* L. plant material used in the thesis obtained from Selçuk University, Faculty of Agriculture, Department of Medicinal Plants.

### 3.1.2. Chemicals

**Table 5.** Chemicals used during the analysis.

| **Name** | **Brand** |
|---|---|
| 1,1,1-Trichloroethane | Sigma-Aldrich |
| 2,4,6-Tribromoanisole | Sigma-Aldrich |
| Acetic acid | Sigma-Aldrich |
| Copper (II) sulfate pentahydrate | Sigma-Aldrich |
| Disodium edta dihydrate | Sigma-Aldrich |
| Ethanol | Sigma-Aldrich |
| Folin & Ciocalteu′ s phenol | Sigma-Aldrich |
| Formaldehyde | Sigma-Aldrich |
| Glutathione reduced | Sigma-Aldrich |
| Glutathione reductase | Sigma-Aldrich |
| Hydrogen peroxide solution | Sigma-Aldrich |
| NADPH | Sigma-Aldrich |
| Phosphate | Sigma-Aldrich |
| Potassium phosphate monobasic | Sigma-Aldrich |
| Sodium azide | Sigma-Aldrich |
| Sodium carbonate | Sigma-Aldrich |
| Sodium dodecyl sulfate | Sigma-Aldrich |
| Sodium hydroxide | Sigma-Aldrich |
| Sodium phosphate | Sigma-Aldrich |
| Sodium tartarate | Sigma-Aldrich |
| Tert-butyl hydroperoxide | Sigma-Aldrich |
| Tris buffer | Sigma-Aldrich |

### 3.1.3. Equipment

**Table 6.** Chemicals used during the analysis.

| **Name** | **Brand** |
|---|---|
| 96-well plate | Corning costar |
| Automated Coverslipper Machine | Leica |
| Balance | Ohaus |
| Blood collecting tubes (including EDTA) | B&D |
| Calculator | Casio |
| Clinical chemistry analyzer | Randox imola |
| Deep freezer (-20 °C) | Siemens |
| Deep freezer (-80 °C) | Aucma |
| Deionized water | ELGA |
| Eppendorf tubes (2 ml) | Isolab |
| Falcons (15 ml) | Isolab |
| Fully auto hematology analyzer | PE-6800 VET |
| Heating and cooling block | Biorad |
| Micropipette | Gilson |
| Microtome device | Rusico |
| Microscope | Olympos |
| Microtome knife Cut-S35 routine | HistoPlus |
| Plate reader | SpectrMax İ3 |
| Quartz cuvette | Agilent |
| Spectrophotometer | Thermo Fisher |
| Tissue staining device | Leica |
| Tissue tracking cassette with cover | HistoPlus |
| Tissue tracking device | Rusico |
| Ultrasonic Bath | Isolab |
| Vortex | Heidolph |
| Grinder | Arçelik |

## 3.2. Methods

### 3.2.1. Experimental Design

The utilization of laboratory animals in this study was subject to a comprehensive ethical assessment and received approval from the Yeditepe University Experimental Animal Ethics Committee, as indicated by Decision Number 14/10/2021 - 2021/10-3. A total of 20 adult male and 20 adult female Sprague Dawley rats were procured from the Yeditepe University Medical Research Center. These animals were housed under standard conditions, including a 12-hour light/dark cycle, and were provided with regular access to food and water. Their living quarters consisted of well-maintained and hygienic cages.

To administer two different dosage levels (665 mg/kg bw/d) for low dose and (2000 mg/kg bw/d) for high dose of processed (debittered) and crude (non-debittered) lupine seeds suspension to the rats via oral gavage, the animals were divided into eight groups, each consisting of five rats, based on their gender. The selection of lupine seeds suspension dosages was guided by previous research and the 2019 assessment report from the EFSA Panel on Contaminants in the Food Chain (CONTAM), which estimated that an average individual would consume 40 grams of lupine daily. To assess potential effects, a dosage three times higher than this was administered to the high-dose groups through oral gavage.

Throughout the 28-day treatment period, the rats were closely observed on a daily basis, and their body weights were systematically recorded every week. The subacute toxicity resulting from chemical exposure was evaluated over this treatment duration. Following the final treatment, the animals were humanely euthanized, and sacrificed. Each animal underwent blood chemistry analyses, and tissue samples from the liver, kidney, and blood were collected and stored at -80°C for subsequent histological examination. Hemogram assays were conducted on blood samples, specifically assessing AST/ALT, Creatinine (CRE), and Urea (URE) levels. Additionally, the animals' weight gain was monitored on a weekly basis.

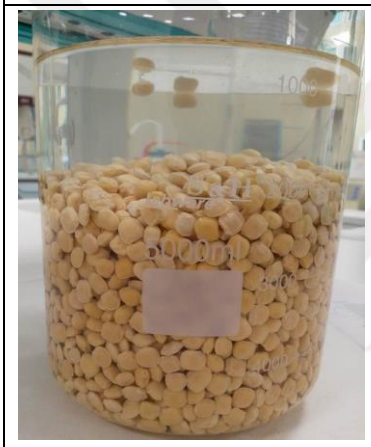
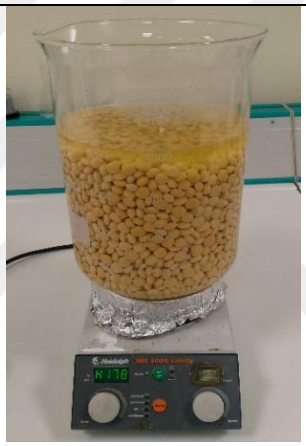
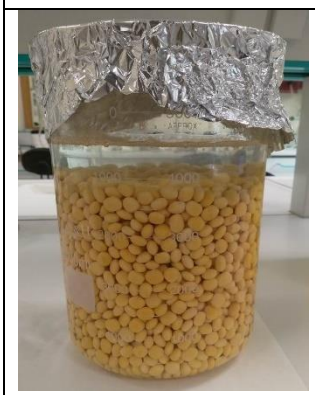
The processed lupine (*Lupinus albus L.*) preparation utilized for animal experiments was meticulously prepared following the method outlined below. Samples, processed similarly to the traditional debittering process, were kept in a dry state to ensure

their suitability for animal experimentation: Preparation and administration stages are shown in Tables 7-11 in below.

1. Lupine Preparation: Initially, foreign matter, damaged, and unsuitable seeds were separated.
2. Soaking: After the cleaning process, the suitable lupine seeds (in a ratio of 1:3, core to water) were soaked in clean distilled water at room temperature (20-25°C) for 4-5 hours.
3. Boiling and Resting: The soaked lupine seeds were then boiled for 60-75 minutes. Subsequently, the water was replaced with clean distilled water at room temperature (20-25°C), and this process was repeated approximately once every 12 hours for a total of 72 hours.
4. Washing: The debittered lupine preparation, obtained by periodically changing the water, was washed with clean water at room temperature (20-25°C) for half a minute.
5. Sodium Chloride (NaCl) Solution: A 6% NaCl solution was prepared using clean distilled water at room temperature (20-25°C).
6. Soaking in NaCl Solution: The washed lupine preparation was immersed in the prepared 6% NaCl mixture for 10-12 hours.
7. Final Washing: The lupine preparation, which had been soaked in the NaCl solution, was washed with clean water at room temperature (20-25°C) for half a minute.
8. Drying: The washed preparation was then dried in a preheated oven at 40°C for approximately 6 hours.
9. Further Drying: Following the oven drying, the preparation's outer coat was removed, and it was further dried by placing it in the shade at room temperature for 1-2 days.
10. Refrigeration: The dried preparation was then stored in a refrigerator at +4 degrees, ensuring it remained ready for use.

This meticulous preparation method was followed to ensure the suitability of the lupine preparation for use in animal experiments.

**Table 7.** Preparation stages of lupine.

| | |
|---|---|
| **#1** Lupine (*Lupinus albus* L.) | **#2** Foreign matter, damaged and unsuitable seeds |
|  |  |
| **#3** (1:3, core: water) kept in clean distilled water | **#4** (1:3, core: water) boiling for 60-75 minutes |
|  |  |
| **#5** Replacing water with clean water at room temperature (20-25C) | **#6** Drying about 6 hours at 40 °C for |
|  |  |

The substances intended for animal testing were pulverized into a fine powder using a household coffee grinder and filtered through a 45-mesh sieve.

**Table 8.** Preparation stages of lupine.

| | |
|---|---|
| **#1** A kitchen type coffee grinder | **#2** Grinding the lupine preparation |
|  |  |
| **#3** Pre-sieving image of ground preparations | **#4** The 45-mesh sieve |
|  |   |
| **#5** Sieving the ground preparation | **#6** Ground and sieved preparation image |
|  |  |

Following the grinding and sieving process, the prepared lupine seeds was suspended in distilled water to create a suspension suitable for use in animal experiments. This suspension was freshly prepared for all experimental groups on a weekly basis and administered daily at the same time throughout the duration of the experiment. It's worth noting that the quantity of the one-week stock preparation was adequate for a 10-day period and was appropriately stored in the refrigerator to maintain its freshness and integrity.

**Table 9.** Dissolving and suspending in distilled water.

| **#1** Preparation in distilled water | **#2** Preparations prepared for all groups |
|---|---|
|  |  |

The Sprague Dawley laboratory animals were procured from YUDETAM (Yeditepe University Faculty of Medicine, Experimental Research Center), and all experimental procedures and animal housing were conducted within the confines of this facility. Throughout the study, the animals were not subjected to relocation from their cages, except for the purposes of periodic weighing and administration of lupine preparations.

To facilitate their well-being, the animals were individually housed in separate cages during the mating period. Furthermore, they were weighed on a weekly basis and received their daily lupine doses, all while having continuous access to both food and water within their respective cages

**Table 10.** Enclosure of animals.

| **#1** Enclosure of animals | **#2** Enclosure of animals |
| --- | --- |
|  |  |

Lupine was administered to the animals using the gavage technique. To ensure the suitability of the pre-made solutions for administration, they were retrieved from the refrigerator and allowed to reach room temperature. Prior to administration, the solutions were thoroughly shaken to prevent any clumping, resulting in a well-mixed suspension.

Following the administration process, any remaining solutions were properly stored in the refrigerator for future use. Importantly, there were no reported issues or complications associated with the passage of the suspension through the Gavage needle during the administration process.

**Table 11.** Administration of the preparation to animals.

| | |
|---|---|
| **#1** Appearance of precipitate taken from the refrigerator | **#2** Suspension after shaking |
|  |  |
| **#3** Giving preparations by gavage method | **#4** Giving preparations by gavage method |
|  |  |

### 3.2.2. Histopathological Analysis

Histopathological analysis is a crucial technique employed to scrutinize tissue specimens under a microscope, facilitating the identification of structural and cellular alterations associated with diseases or injuries. The procedure encompasses several key steps, enabling the detection of abnormal cellular growth, tissue impairments, and specific

pathological conditions. Here is an outline of the typical process involved in histopathological analysis:

1. Tissue Sampling: Tissue specimens are acquired via methods such as biopsy or surgical excision.
2. Tissue Preservation: To preserve tissue integrity and prevent degradation, the samples are immersed in formalin.
3. Tissue Embedding: The preserved samples are embedded in paraffin wax and subsequently sliced into thin sections utilizing a microtome.
4. Staining: The thin tissue sections are stained with specialized dyes, such as Hematoxylin and Eosin (H&E), which enhance the visibility of various cell types and structures.
5. Microscopic Examination: A pathologist thoroughly examines the stained tissue sections under a microscope, meticulously scrutinizing them for any anomalous changes or signs of diseases.
6. Reporting and Diagnosis: Based on their observations, the pathologist generates a comprehensive report detailing their findings, including a diagnosis when applicable.

In our experiments, the management of tissue samples involved placing them in sealed embedding cassettes. These cassettes were then incorporated into a tissue tracking system, and the samples were embedded in paraffin. Subsequently, the paraffin-embedded tissues were sectioned using a microtome and affixed onto microscope slides, which were then covered with 24x60 mm coverslips. To facilitate microscopic examination for cellular damage, the tissue slides were subjected to the hematoxylin-eosin staining procedure. Any identified cellular damage was assessed and graded using statistical methods to gain further insights into the experimental outcomes.

#### 3.2.2.1. Tissue Samples Tapering

Tissue sample tapering is a technique that involves reducing the size of a tissue specimen, shaping it into a thin and pointed form. This procedure is typically carried out using a specialized tool known as a microtome, which employs a sharp blade to make precise cuts in the tissue sample. Tapered tissue samples find utility in various applications, including staining and microscopic examination.

In our experiments, tissue samples extracted from the liver and kidneys were immersed in a solution of 10% neutral formaldehyde derived from animals, with a

concentration of 0.1 M. Notably, the kidney samples were divided into two parts, a step taken to enhance the absorption of chemicals during the experimental process.

#### 3.2.2.2. Organization of Devices

In experimental setup, several specialized tools used and equipment for tissue processing and analysis:

1. Tissue Tracking Device: This device featured 11 compartments and was utilized in conjunction with Program 3 for efficient tracking and management of tissue samples.
2. Neutral Formaldehyde Solution: a neutral formaldehyde solution prepared by combining 0.1 M phosphate buffer with 37% formaldehyde sourced from Sigma-Aldrich. This solution was employed for tissue preservation.
3. Tissue Embedding Machine: To create liquid paraffin, solid paraffin was heated to 65°C using a tissue embedding machine. A small quantity of the liquid paraffin was then added to the sample cup, and pressure was applied to secure the tissue specimen.
4. Freezing Process: The sample cups, containing the tissue secured with liquid paraffin, were filled with liquid paraffin and subjected to freezing. Dry ice was employed above the device to facilitate the freezing process.
5. Microtome: A microtome utilized for making precise cuts in the tissue samples that had been embedded in paraffin. Specifically, a disposable microtome blade was used for this purpose.
6. Tissue Sectioning: To create appropriate tissue sections, the sections were heated with hot water and then promptly placed onto microscope slides, ensuring their readiness for subsequent analysis and examination under a microscope.

#### 3.2.2.3. Tissue Staining

Tissue staining is a crucial technique used in biological and medical research to enhance the visibility of specific aspects within biological tissues. This method is instrumental in helping scientists and medical professionals better examine and understand the structure and function of cells and tissues. Various staining techniques can be employed to highlight different cellular structures, such as cell nuclei, cell membranes, or specific proteins. One widely used staining method is Hematoxylin and Eosin (H&E) staining, which imparts a blue color to nuclei and a pink color to cytoplasm, facilitating the clear differentiation of various cells and tissue structures.

Here is a detailed explanation of the Hematoxylin-Eosin (H&E) staining process:

1. Deparaffinization: Tissue samples are initially immersed in xylene for 10 minutes to remove paraffin.
2. Dehydration: The samples are then placed in progressively more concentrated ethanol solutions for 10 minutes each to dehydrate the tissue.
3. Hematoxylin Staining: Tissue samples are immersed in a hematoxylin solution for 5-6 minutes, followed by a rinse in distilled water. An acid alcohol solution is applied to create a light blue color, followed by another rinse.
4. Eosin Staining: The samples are then immersed in an eosin solution for 3-4 minutes and rinsed in ethanol solutions of decreasing concentration.
5. Final Dehydration: The samples are placed in absolute ethanol for 15-20 minutes.
6. Clearing: Subsequently, the samples are placed in xylene for 10 minutes, repeated three times. This process changes the color of the cytoplasm to pink-red, the nucleus to dark blue-violet, and the cytoplasm to sky blue.

For the second portion of the kidney samples, the Periodic Acid-Schiff (PAS) Staining Method was employed using an automatic apparatus and program 3. The PAS technique involves the following steps:

1. Periodic Acid Solution Preparation: A solution is made by dissolving 2 grams of periodic acid in 400 mL of distilled water.
2. Schiff Solution Preparation: In an Erlenmeyer flask, 2 grams of Basic Fuchsin are dissolved in 400 mL of distilled water and heated to boiling on a hot plate. After cooling, 4 grams of potassium metabisulfite are added when the solution reaches 50°C, and it is further cooled to room temperature. Subsequently, 4 mL of HCl and 4 grams of activated carbon are added, and the solution is left to sit overnight.

The staining process for tissue samples involves immersion in a periodic acid solution for 5 minutes to remove paraffin, followed by incubation in Schiff solution for 15 minutes. The samples are then washed, stained with Hematoxylin, and rinsed with distilled water and ethanol. The final step involves clearing the samples with xylene in WASH 2, resulting in the change of color of glycogen and carbohydrates from red to purple, and the nucleus from red to blue. Automated staining devices, such as the one used in this process, follow specific programs for each staining method and are designed to streamline the staining process efficiently. Finally, an automatic coverslip closing system is utilized to cover the stained samples with 24x60mm coverslips in approximately

50 minutes. This comprehensive staining process allows for the detailed examination of tissue samples under a microscope, aiding in research and diagnosis.

### 3.2.3. Measurement of Protein Levels

The Lowry method is a commonly employed technique for quantifying protein concentration in a blood sample. This method relies on the interaction between copper ions and the peptide bonds present in proteins, particularly in an alkaline environment. This interaction results in the reduction of copper ions to Cu+1, which subsequently leads to the formation of a colored product when reacting with the Folin-Phenol reagent. The procedure involves several key steps:

1. Sample Preparation: Blood samples, standard solutions of known protein concentration, and a blank (containing distilled water) are each added to separate tubes.
2. Addition of Reagent C: Reagent C is added to all tubes, and the mixture is allowed to stand at room temperature for approximately 30 minutes.
3. Introduction of Folin-Phenol Reagent: Folin-Phenol reagent (300 µL) is added to all tubes, and the contents are thoroughly mixed (e.g., vortexed). The tubes are then left at room temperature for about 45 minutes.
4. Measurement of Absorbance: The absorbance of the contents in all tubes is measured against the blank at a wavelength of 660 nm using a spectrophotometer.
5. Calculating Protein Levels: Protein concentrations in the samples are determined by referencing the absorbance readings to a standard calibration chart. This chart correlates known protein concentrations with their corresponding absorbance values.

The Lowry method is a reliable technique for quantifying protein concentrations in samples. However, it's important to note that while this method provides a measure of reducing substances, it is not specific solely to proteins and may detect other compounds with reducing properties.

### 3.2.4. Measurement of MDA Levels

Measuring the levels of MDA (Malondialdehyde) in a blood sample can be achieved through the Thiobarbituric Acid Reactive Substances (TBARS) assay. This method is designed to detect the reaction between MDA and thiobarbituric acid, forming a colored product that can be quantified. MDA levels in the blood sample are typically reported in units of micromoles per liter (µmol/L). It's important to note that MDA is not

a specific marker for any particular disease but serves as a general indicator of oxidative stress and inflammation.

Here's a description of the procedure used to measure MDA levels in homogenates, adapted for use in 96-well plates:

1. Sample Preparation: 15 µL of each sample is combined with 15 µL of SDS (8.1%), 112.5 µL of acetic acid (20%), 112.5 µL of TBA (0.8%), and deionized water.
2. Heating and Incubation: The mixture is heated at 95 °C using a plate warming and cooling block for one hour.
3. TCA Addition: After incubation, 150 µL of a combination of TCA (Trichloroacetic acid) and the reaction mixture are added in equal amounts.
4. Centrifugation: The plate is then spun at 1000x g for 10 minutes.
5. Detection and Measurement: The test detects "the formation of red addition reactions in acidic media between TCA and MDA," and this reaction is quantified by measuring absorbance at 532 nm using a spectrophotometer.
6. The concentration of MDA is reported in nanomoles per gram (nmol/g) of protein, with a tetra methoxy propane solution used as a reference for calibration. This assay allows for the quantification of MDA levels in the samples, providing insights into oxidative stress and related processes.

### 3.2.5. Determination of GSH-Px Activity

The activity of Glutathione Peroxidase (GSH-Px), an enzyme crucial for protecting cells from oxidative stress by converting hydrogen peroxide and organic hydroperoxides into water and alcohols, can be determined using various methods. One commonly used method involves measuring the rate at which glutathione (GSH) is oxidized to form glutathione disulfide (GSSG) in the presence of a peroxide substrate. This reaction can be monitored by measuring the change in absorbance at 340 nm using a spectrophotometer. The activity of GSH-Px is typically reported in units per milligram of protein or per milliliter of the sample.

Here's a detailed description of the procedure used to determine GSH-Px activity:

1. Reagent Preparation: Prepare a solution containing 1 mmol/L sodium EDTA, 2 mmol/L reduced GSH, 0.2 mmol/L NADPH, 4 mmol/L sodium azide, and 1000 U GSH-Rd in 50 mmol/L Tris buffer (pH 7.6).

2. Sample Preparation: Dilute cortex homogenates three times before the experiment.

3. Experimental Setup: In a quartz cuvette, combine 10 mL of the sample and 990 mL of the prepared reagent mixture. Incubate the cuvette at 37 °C for 5 minutes.

4. Initiating the Reaction: Add 10 μL of tert-butyl hydroperoxide solution to start the reaction (dilution ratio: 1:1000).

5. Monitoring Absorbance: Observe the decrease in NADPH absorbance at 340 nm for 3 minutes. Measure the absorbance of each sample at 30-second intervals.

6. Calculating GSH-Px Activity: Determine the difference in absorbance per minute for each sample.

7. Reporting Activity: Report the activity of GSH-Px as units per gram (U/g) of protein.

This method allows for the quantification of GSH-Px activity, providing insights into the enzyme's role in protecting cells from oxidative stress.

### 3.2.6. Determination of URE, CREA, AST, ALT Levels

The "Determination of URE, CREA, AST, ALT Levels" refers to the process of quantifying the levels of four specific substances in a sample. These substances are urea, creatinine, aspartate aminotransferase (AST), and alanine aminotransferase (ALT). Measuring these substance levels can provide valuable insights into the functioning of the kidneys and liver. Abnormal levels of these substances may indicate the presence of diseases or injuries affecting these organs. Various methods can be employed to measure these substance levels, including blood and urine tests, as well as enzymatic assays. These tests are instrumental in detecting any dysfunction or damage within the kidneys and liver.

For the quantitative in vitro determination of urea concentration, creatinine, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels, test systems from Randox are utilized in conjunction with the RX imola device. This combination of reagents and instrumentation allows for precise and accurate measurements of these critical substances, aiding in the assessment of kidney and liver health.

#### 3.2.6.1. UREA

Urea, scientifically referred to as carbamide, is a waste product generated during the liver's breakdown of proteins. Subsequently, it is transported to the kidneys, where it

is excreted from the body through urine. Elevated levels of urea in the bloodstream, medically known as hyperuremia, can potentially signify kidney malfunction or dehydration. Conversely, reduced levels of urea in the blood, termed hypouremia, may suggest liver ailments or an accelerated protein metabolism.

Blood tests, such as the BUN (blood urea nitrogen) test, are routinely employed to measure urea levels, aiding in the assessment of kidney functionality. These tests help in the identification and management of kidney and metabolic disorders. Urea undergoes decomposition upon contact with water, facilitated by an enzyme called urease, leading to the formation of ammonia and carbon dioxide. Furthermore, the ammonia produced in this process interacts with alpha-oxoglutarate, NADH, and an enzyme called glutamate dehydrogenase, ultimately yielding glutamate and $NAD^+$. [281]

#### 3.2.6.2. CREA

CREA likely pertains to creatinine, a byproduct stemming from muscle metabolism that is detectable in the bloodstream. Elevated creatinine levels can potentially signify kidney injury or suboptimal renal function. Evaluating creatinine levels constitutes a routine component of blood assessments, frequently conducted concurrently with other renal health markers such as BUN and eGFR. The colorimetric technique is employed for quantifying creatinine. Creatinine, a nitrogenous waste derivative, originates from creatine and creatine phosphate found within muscle tissue. In contrast to various substances, creatinine is not subject to recycling and is expelled from the body via urine, facilitated by the kidneys. The rate of creatinine generation and elimination remains stable and directly correlates with the volume of muscle mass in the body. Because of the renal elimination process, creatinine primarily serves as an indicator to assess kidney functionality. Creatinine is regarded as the most dependable parameter for diagnosing and managing kidney-related conditions. Its measurement primarily aims to appraise renal function and offers distinct advantages over urea measurement. Importantly, creatinine levels in the bloodstream remain unaffected by factors such as protein intake, fluid volume, urine output, and physical activity. Elevated blood creatinine levels indicate impaired kidney clearance, implying potential renal function issues. Conversely, diminished creatinine levels are rare and generally lack clinical significance. [282]

#### 3.2.6.3. AST

AST, short for Aspartate Aminotransferase, is an enzyme predominantly situated in the liver but is also present in other organs like the heart and skeletal muscles. It is typically included in liver function assessments, alongside enzymes such as ALT (Alanine Aminotransferase) and ALP (Alkaline Phosphatase). Elevated levels of AST may serve as an indicator of liver damage or diseases such as viral hepatitis, cirrhosis, or liver cancer. Nevertheless, heightened AST levels can also be associated with conditions unrelated to the liver, such as heart attacks or muscle injuries. Consequently, AST levels alone cannot definitively diagnose liver ailments and should be evaluated in conjunction with additional clinical and laboratory findings. [283]

The ultraviolet method is employed to measure AST (Aspartate Aminotransferase), an enzyme belonging to a group responsible for modifying amino acids and alpha-oxoacids by transferring amino groups. AST is present in both the cytoplasm and mitochondria of cells. In instances of mild liver damage, the primary AST form in the bloodstream originates from the cytoplasm, with a smaller proportion from the mitochondria. However, during severe damage, more mitochondrial enzymes are released. Elevated AST levels can signify myocardial infarction, liver disorders, muscular dystrophy, and damage to other organs. To standardize AST measurements, the International Federation of Clinical Chemistry (IFCC) has established guidelines, including specific buffer and substrate concentrations, the preference for Tris buffers over phosphate buffers, pre-incubation of the buffer and blood sample to account for side reactions, and the use of alpha-oxoglutarate as the substrate. This standardized method has been optimized according to IFCC recommendations. The test gauges the interaction between alpha-oxoglutarate and L-aspartate in the presence of AST, resulting in the production of L-glutamate and oxaloacetate. The amount of NADH consumed is subsequently utilized to determine the AST level in the sample. [283]

#### 3.2.6.4. ALT

Alanine transaminase (ALT) is an enzyme found primarily in the liver, but also in other organs such as the kidney and pancreas. It plays a role in the body's metabolism of amino acids, specifically in the transfer of an amino group from alanine to alpha-ketoglutarate, producing pyruvate and glutamate. Elevated levels of ALT in the blood can indicate liver damage or disease, such as hepatitis or cirrhosis. However, certain

conditions like muscle injury, certain medications and alcohol consumption also can cause an increase in ALT levels. Measuring ALT levels through a blood test is commonly used as a marker of liver function. [284]

The UV method involves the use of enzymes called aminotransferases to convert amino acids and alpha-oxoacids by transferring amino groups. One specific enzyme, ALT, is found in high concentrations in the liver and in decreasing concentrations in other organs such as the kidney, heart, and muscles. The measurement of ALT levels is used to diagnose and treat liver and heart diseases, and high levels of transaminases can indicate damage to the heart, liver, muscles, and other organs. Elevated levels of ALT in the blood are mostly associated with liver disease. The IFCC (International Federation of Clinical Chemistry) has established a standardized procedure for measuring ALT levels in the blood. This procedure includes optimizing the concentration of substrates, using Tris buffers instead of phosphate, pre-incubating the buffer and serum to allow for side reactions with NADH, using α-oxoglutarate as the substrate, and including an optional step of activating pyridoxal phosphate. This is considered to be an optimized standard method as per the recommendations of the IFCC. In this method, ALT catalyzes the reaction between α-oxoglutarate and L-alanine to produce L-glutamate and pyruvate. The indicator reaction used to measure NADH consumption is based on the presence of pyruvate. [284]

### 3.2.7. Determination of Hemogram Parameters

Hematology constitutes a specialized medical field dedicated to the comprehensive study of blood and blood-related organs. The assessment of hematology levels entails the examination of various components within blood cells, including red blood cells, white blood cells, and platelets. This evaluation is typically carried out using a complete blood count (CBC) test, which furnishes vital information regarding the quantity and characteristics of these blood constituents. Furthermore, supplementary tests like a blood smear can be employed to scrutinize the morphology and size of blood cells.

In-depth hematology analysis encompasses the assessment of key parameters such as hemoglobin levels, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin. These measurements hold immense diagnostic and therapeutic significance in addressing a spectrum of blood-related conditions, including but not limited to anemia, leukemia, and thrombocytopenia.

To facilitate these assessments, cutting-edge technology such as the PE-6800 fully automatic hematology analyzer is deployed. This advanced instrument enables the quantitative in vitro analysis of 20 distinct parameters and generates three histograms based on blood specimens, thereby offering comprehensive insights into the hematological profile. The blood parameters are given in Table 12 below.

**Table 12.** Blood parameters with abbreviations and units.

| Item | Abbreviation | Unit |
|---|---|---|
| White blood cell or leukocyte | WBC | $10^9$/L |
| Lymphocyte | LYM# | $10^9$/L |
| Mid-sized cell | MID# | $10^9$/L |
| Granulocyte | GRAN# | $10^9$/L |
| Lymphocyte percentage | LYM% | % |
| Mid-sized cell percentage | MID% | % |
| Granulocyte percentage | GRAN% | % |
| Red Blood Cell or erythrocyte | RBC | $10^{12}$/L |
| Hemoglobin Concentration | HGB | g/L |
| Hematocrit | HCT | % |
| Mean Corpuscular(erythrocyte) Volume | MCV | fL |
| Mean Cell Hemoglobin | MCH | pg |
| Mean Cell Hemoglobin Concentration | MCHC | g/L |
| Red Blood Cell Distribution Width- Standard Deviation | RDW-SD | fL |
| Red Blood Cell(erythrocyte) Distribution Width Coefficient of Variation | RDW-CV | % |
| Platelet | PLT | $10^9$/L |
| Mean Platelet Volume | MPV | fL |
| Platelet Distribution Width | PDW | % |
| Plateletcrit | PCT | % |
| Plateletcrit-large Cell Ratio | P-LCR | % |
| White Blood Cell Histogram | WBC Histogram | |
| Red Blood Cell Histogram | RBC Histogram | |
| Platelet Histogram | PLT Histogram | |

### 3.2.8. Statistical Analysis

The data underwent analysis using the SPSS 28.0 software (Statistical Packages of Social Sciences) on a computer. Descriptive statistics were provided, encompassing measures such as the mean ± standard deviation, median, as well as the minimum and maximum values for continuous variables. To assess the adherence of the data to a normal distribution, the Shapiro-Wilk test was conducted. Furthermore, the equality of variances among groups was assessed using Levene's test. For variables that exhibited both normal distribution and homogeneous variances among the groups, the ANOVA test was employed to make comparisons. In cases where statistically significant differences were observed, the Tukey test was subsequently applied as a post-hoc analysis for pairwise comparisons. Conversely, for variables displaying normal distribution but non-homogeneous variances across groups, the Welch ANOVA test was utilized. In situations where the data did not conform to a normal distribution, the Kruskal-Wallis test was employed to compare variables among the groups. Additionally, Bonferroni correction was employed to adjust the p-values. Significance was considered at a threshold of $p<0.05$.

The Shapiro-Wilk test is a statistical tool used to determine if a dataset follows a normal distribution. It works by comparing a sample of data to the expected normal distribution and generates a p-value based on the disparities between the sample and the expected normal distribution. When the p-value is less than a predetermined threshold, usually 0.05, it indicates that the data does not conform to a normal distribution. It's important to note that the test's ability to detect deviations from normality improves with larger sample sizes.

On the other hand, Levene's test provides a way to assess whether the variances among two or more groups are similar. It involves comparing the variances across these groups and calculating a p-value based on the differences observed in the variances. If the computed p-value is below a predefined threshold, typically 0.05, it suggests that the variances among the groups are not equal. Notably, Levene's test can serve as a viable alternative to the commonly used F-test for assessing variance equality, especially when the data doesn't follow a normal distribution.

ANOVA, short for Analysis of Variance, is a statistical technique employed to examine whether there are statistically noteworthy distinctions among the means of two or more groups. It accomplishes this by assessing the group means and determining a p-

value grounded in the variations among these means. If the obtained p-value falls below a predetermined threshold, typically 0.05, it suggests that at least one of the means is dissimilar from the others. ANOVA can be utilized for both one-way analysis, which evaluates multiple groups with a single independent variable, and multi-way analysis, which assesses multiple groups with multiple independent variables. It is crucial to ensure that the data adheres to the assumptions of normality and equal variances before applying ANOVA; otherwise, alternative methods such as Welch's ANOVA or the Kruskal-Wallis test may be more appropriate.

Welch's ANOVA is a variation of the traditional Analysis of Variance (ANOVA) test used to ascertain if there are significant disparities among the means of two or more groups when the group variances are not uniform. In contrast to standard ANOVA, which assumes uniform group variances, Welch's ANOVA provides a robust alternative for cases where this assumption doesn't hold.

The Kruskal-Wallis test is a non-parametric technique for comparing multiple groups of ordinal or continuous data, without making assumptions about normality or equal variances. It serves a similar purpose to the one-way ANOVA test but is particularly useful when these assumptions are not met.

# 4. RESULTS

## 4.1. Body, Liver and Kidney Weights of Rats

After the rats had become accustomed to the controlled laboratory setting, their body masses were measured at intervals spanning the first, second, third, and fourth weeks. The animal groups are given in Table 13 below and will be stated with their group codes from now on. The alterations in animal weights were recorded and are presented in Table 14 over a four-week period.

**Table 13.** Groups used during experiments and their group codes.

| **Group Name** | **Group Code** |
|---|---|
| A - Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d) | A |
| B - Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d) | B |
| C - Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d) | C |
| D - Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d) | D |
| E - Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d) | E |
| F - Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d) | F |
| G - Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d) | G |
| H - Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d) | H |

**Table 14.** The body weights of the rats within each respective experimental group.

| Groups | Body Weight (g), Weight Change Compared to Previous Weeks (%) | | | |
|---|---|---|---|---|
| | **Week 1** | **Week 2 (vs Week 1 %)** | **Week 3 (vs Week 2 %)** | **Week 4 (vs Week 3 % & vs Week 1 %)** |
| A | 281,20 ± 26,69 | 285,60 ± 24,09 (2%) | 311,20 ± 26,46 (9%) | 303,80 ± 27,31 (-2%) (8%) |
| B | 175,00 ± 11,15 | 179,40 ± 10,48 (3%) | 188,80 ± 8,32 (5%) | 190,60 ± 8,40 (1%) (9%) |
| C | 236,40 ± 21,50 | 250,20 ± 24,12 (6%) | 278,40 ± 18,75 (11%) | 273,20 ± 18,35 (-2%) (16%) |
| D | 183,80 ± 5,91 | 184,20 ± 7,30 (0%) | 202,40 ± 7,76 (10%) | 201,80 ± 3,96 (0%) (10%) |
| E | 249,40 ± 13,70 | 268,40 ± 11,72 (8%) | 296,80 ± 11,72 (11%) | 283,60 ± 26,64 (-4%) (14%) |
| F | 180,20 ± 4,70 | 189,20 ± 9,32 (5%) | 197,40 ± 6,374 (4%) | 196,60 ± 8,26 (0%) (9%) |
| G | 269,00 ± 18,20 | 293,00 ± 16,11 (9%) | 304,60 ± 12,75 (4%) | 316,20 ± 13,13 (4%) (18%) |
| H | 190,60 ± 14,23 | 200,40 ± 13,30 (5%) | 202,40 ± 12,64 (1%) | 207,20 ± 6,910 (2%) (9%) |

The data is displayed as the mean plus or minus the standard deviation (mean ± SD).

A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

The final data analysis involved calculating the average body weight results from weeks 1, 2, 3, and 4 shown in Figure 25 below. When considering the average weights of rats in both the first and fourth weeks, it became evident that increased body weight was observed across all experimental groups.

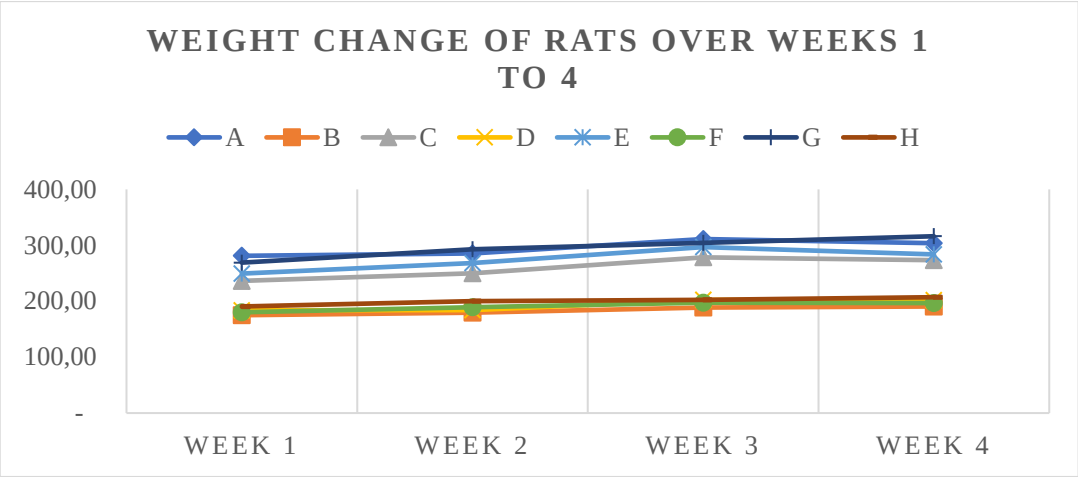



**Figure 25.** The alteration in the weights of the rats over the course of weeks 1 to 4. A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

At the conclusion of the study, measurements were conducted to determine the weights of the rats' livers and kidneys. Table 15 displays the average liver and kidney weights as ratios to body weights, alongside their corresponding means and standard deviations (mean ± SD). Figure 26 and 27 shows the liver and kidney weights of the male and female groups compared to their respective body weights in below.

**Table 15.** The weights of the body, liver, and kidneys, as well as their respective ratios to the body weights, were assessed for the rats in each experimental group.

| Group | Average Body Weight (g) | Liver Weight (g) | Kidney Weight (g) | Liver/Body Weight (g) Ratio | Kidney/Body Weight (g) Ratio |
|---|---|---|---|---|---|
| **A** | 295,45 ± 26,09[1] | 11,58 ± 1,03 | 3,05 ± 0,32 | 0,04 | 0,01 |
| **B** | 183,45 ± 9,98 | 7,83 ± 0,61 | 1,80 ± 0,10 | 0,04 | 0,01 |
| **C** | 259,55 ± 22,44[1, 2] | 10,07 ± 1,26 | 2,80 ± 0,25 | 0,04 | 0,01 |
| **D** | 193,05 ± 6,63 | 8,01 ± 0,30 | 1,94 ± 0,17 | 0,04 | 0,01 |
| **E** | 274,55 ± 15,32 | 10,21 ± 1,14 | 2,97 ± 0,25 | 0,04 | 0,01 |
| **F** | 190,85 ± 7,08 | 8,00 ± 0,86 | 2,12 ± 0,23 | 0,04 | 0,01 |
| **G** | 295,70 ± 15,5[2] | 11,2 ± 0,92 | 2,99 ± 0,35 | 0,04 | 0,01 |
| **H** | 200,15 ± 12,47 | 8,75 ± 0,66 | 2,05 ± 0,14 | 0,04 | 0,01 |

The data is displayed as the mean plus or minus the standard deviation (mean ± SD).

A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

[1]There is a significant difference between A and C, with a p-value less than 0.05. The average weight of Group A is higher than that of Group C.

[2]There is a statistically significant difference between G and C, with a p-value below 0.05. The average weight of Group G exceeds that of Group C.

In general, among the male groups, those administered a high dose of processed (debittered) lupine seeds suspension exhibited a greater increase in weight compared to the group receiving crude (unbittered) lupine seeds suspension. Conversely, when considering only groups that received crude (unbittered) lupine seeds suspension, the low-dosage group demonstrated a higher weight gain. Based on the results obtained from the female groups, no statistically significant differences were observed among the groups.

The results of the liver and kidney weight analysis suggest that there is no statistically significant gender-based difference between the two groups, as indicated by the findings for both males and females.

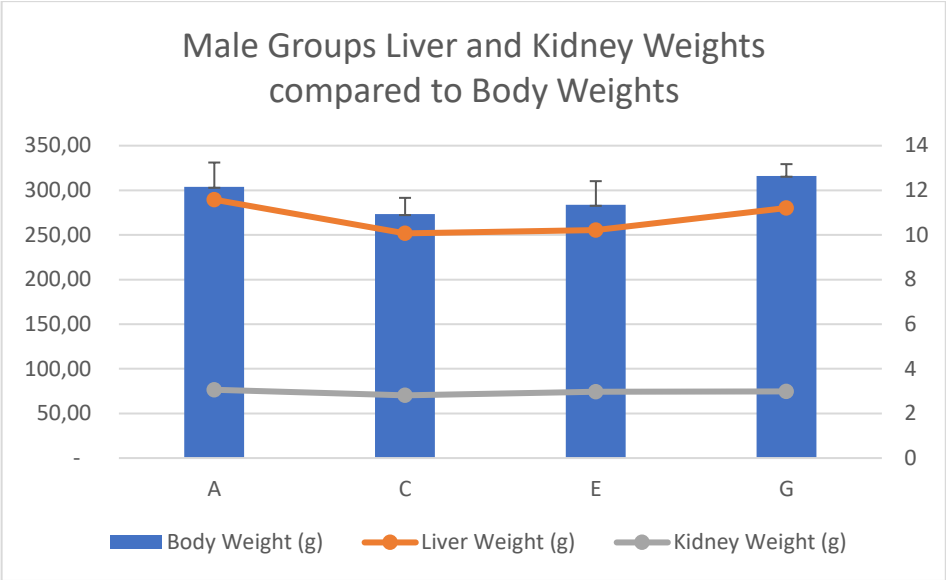



**Figure 26.** The liver and kidney weights of the male groups were compared to their respective body weights. A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d);

There were no significant differences observed in the average liver and kidney weight ratios to body weight.

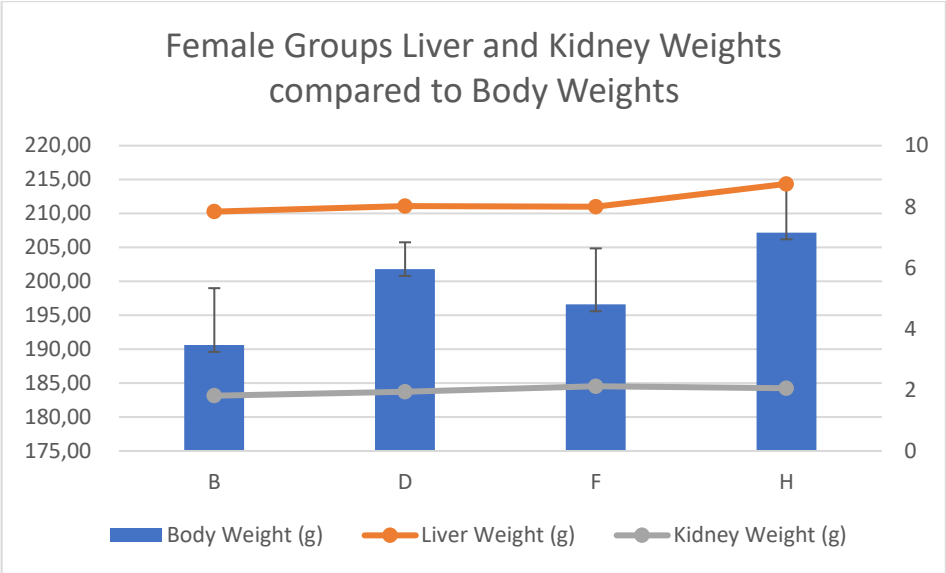



**Figure 27.** The liver and kidney weights of the female groups were compared to their respective body weights. B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

There were no significant differences observed in the average liver and kidney weight ratios to body weight.

## 4.2. Hemogram Values of Rats

Table 16 provides a depiction of the hemogram values obtained from blood samples collected from the rats on the last day of the experimental procedure. Figure 28-31 shows respectively WBC, RBC, HGB and HCT the values for rats in experimental groups.

**Table 16.** Hemogram values for rats in the experimental groups.

| Groups | WBC (10^3/µL) | WBC Range Alarm | RBC (10^6/µL) | RBC Range Alarm | HGB (g/dL) | HGB Range Alarm | HCT % | HCT Range Alarm |
|---|---|---|---|---|---|---|---|---|
| **A** | 10,36 ± 5,64 | | 9,67 ± 1,30 | | 23,58 ± 4,28 | | 60,34 ± 8,55 | |
| **B** | 9,02 ± 7,58 | | 5,74 ± 4,71 | | 14,24 ± 11,73 | | 36,48 ± 29,93 | |
| **C** | 14,78 ± 3,40 | | 9,86 ± 0,28 | | 24,26 ± 0,96 | | 62,60 ± 2,47 | |
| **D** | 13,96 ± 1,21 | 3.0 - 17.0 | 9,05 ± 0,63 | 5.0 - 10.0 | 22,16 ± 1,72 | 11.0 - 19.0 | 58,02 ± 4,36 | 35.0 - 45.0 |
| **E** | 20,46 ± 3,36[1] | | 9,44 ± 0,80 | | 23,74 ± 2,16 | | 61,08 ± 3,86 | |
| **F** | 11,14 ± 1,69[1] | | 9,50 ± 0,41 | | 23,24 ± 1,29 | | 61,02 ± 1,90 | |
| **G** | 16,20 ± 0,09 | | 8,87 ± 0,83 | | 22,25 ± 1,55 | | 55,35 ± 6,05 | |
| **H** | * | | * | | * | | * | |

WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit. The data is displayed as the mean plus or minus the standard deviation (mean ± SD). *Tests could not be performed due to equipment failure.

A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

[1]There is a significant difference between for WBC levels of E and F, with a p-value less than 0.05. The average WBC of Group E is higher than that of Group F.

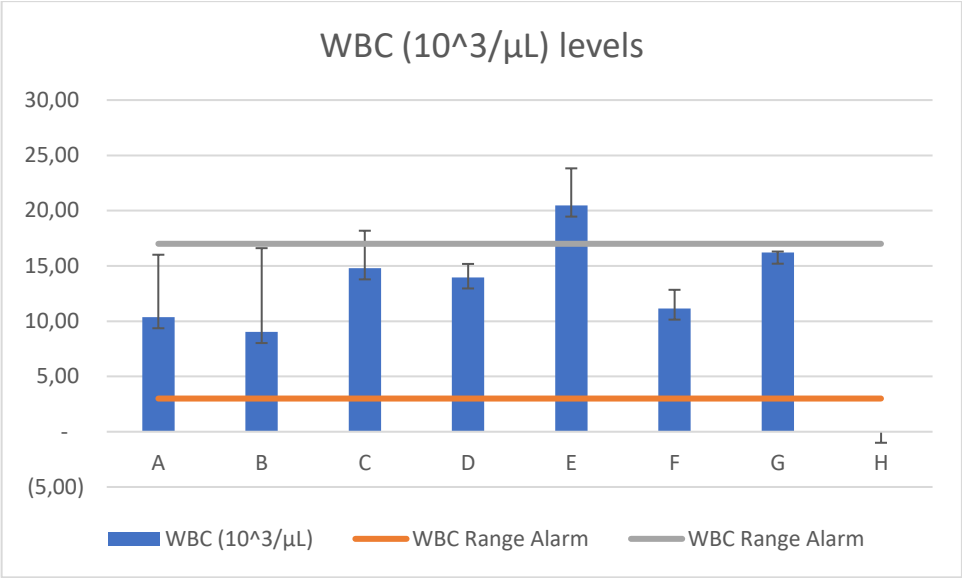



**Figure 28.** WBC values for rats in the experimental groups. WBC, white blood cell; A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

The increase in WBC values was observed exclusively in the "E" group, with a significant difference between E and F, with a p-value less than 0.05. The average WBC of Group E is higher than that of Group F.

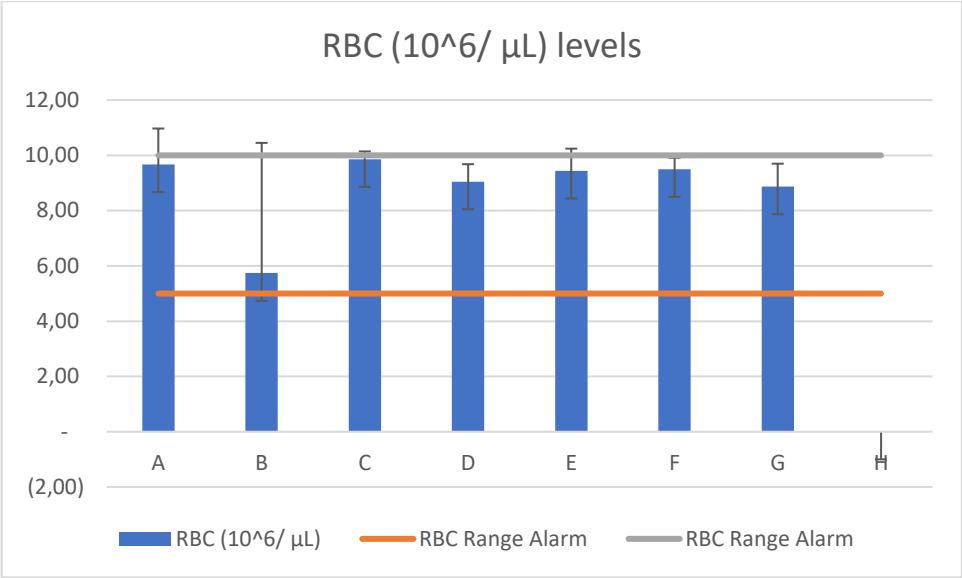



**Figure 29.** RBC values for rats in the experimental groups. RBC, red blood cell; A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

RBC values remained within acceptable ranges for all groups.

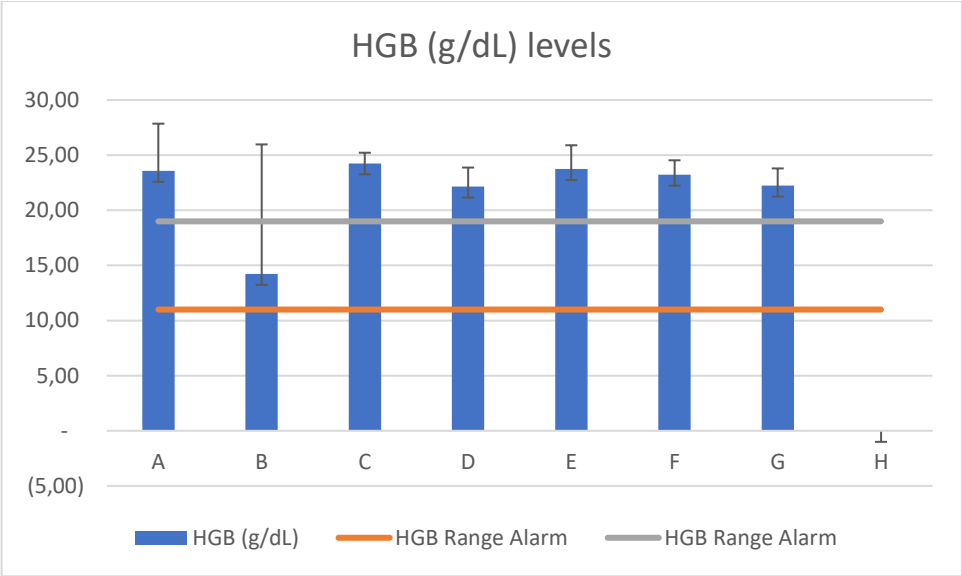



**Figure 30.** HGB values for rats in the experimental groups. HGB, hemoglobin; A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

HGB values could potentially be attributed to dehydration, while not showing any statistically significancy.

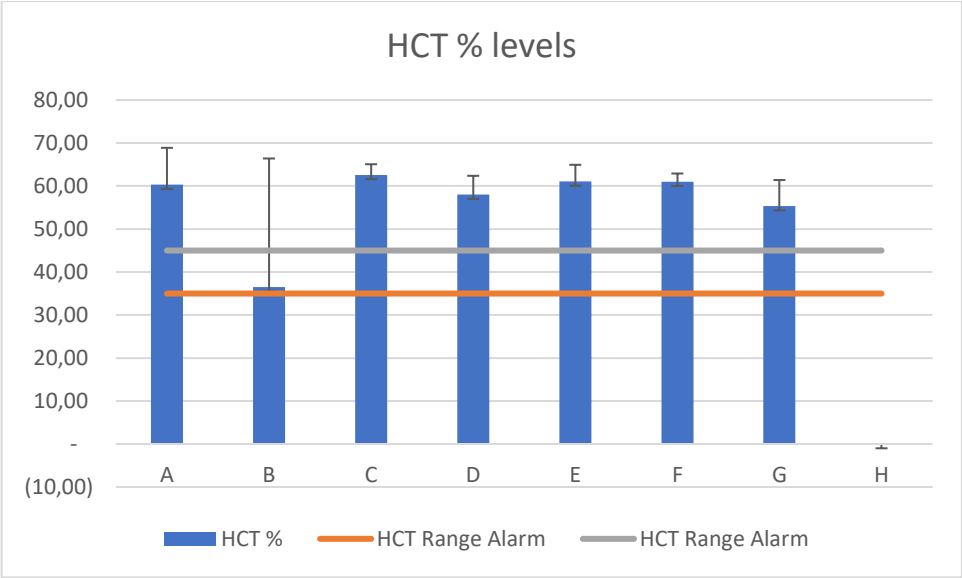



**Figure 31.** HCT values for rats in the experimental groups. HCT, hematocrit; A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

HCT values could potentially be attributed to dehydration, while not showing any statistically significancy.

### 4.3. Liver and Kidney Function Test Values of Rats

Table 17 presents the hemogram values and liver and kidney function test results obtained from rat blood samples collected on the final days of the experimental procedure. Figure 32 and 33 shows Liver and Kidney function test values for male and female groups respectively.

**Table 17.** Liver and Kidney function test values for rats in the experimental groups.

| Groups | ALT | AST | CRE | URE |
|---|---|---|---|---|
| **A** | 90,20 ± 12,82 | 127,00 ± 24,24 | 0,66 ± 0,22 | 39,20 ± 2,78 |
| **B** | 98,20 ± 7,52 | 106,60 ± 20,90 | 0,59 ± 0,20 | 41,20 ± 3,65 |
| **C** | 90,60 ± 22,33 | 129,00 ± 44,57 | 0,48 ± 0,18 | 38,80 ± 2,99 |
| **D** | 97,00 ± 13,03 | 182,80 ± 89,69 | 0,58 ± 0,04 | 40,00 ± 2,44 |
| **E** | 95,00 ± 10,37 | 125,60 ± 22,35 | 0,58 ± 0,05 | 37,60 ± 3,20 |
| **F** | 71,60 ± 14,56[1] | 117,40 ± 20,36 | 0,58 ± 0,16 | 40,40 ± 4,58 |
| **G** | 101,80 ± 6,11[1] | 164,20 ± 137,44 | 0,59 ± 0,09 | 36,80 ± 1,32 |
| **H** | 82,80 ± 4,62 | 99,60 ± 12,81 | 0,63 ± 0,13 | 34,80 ± 1,46 |

ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; CREA, Creatinine; URE, Urea. The data is displayed as the mean plus or minus the standard deviation (mean ± SD).

A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

[1]There is a significant difference for ALT levels of G and F, with a p-value less than 0.05. The average ALT level of Group G is higher than that of Group F.

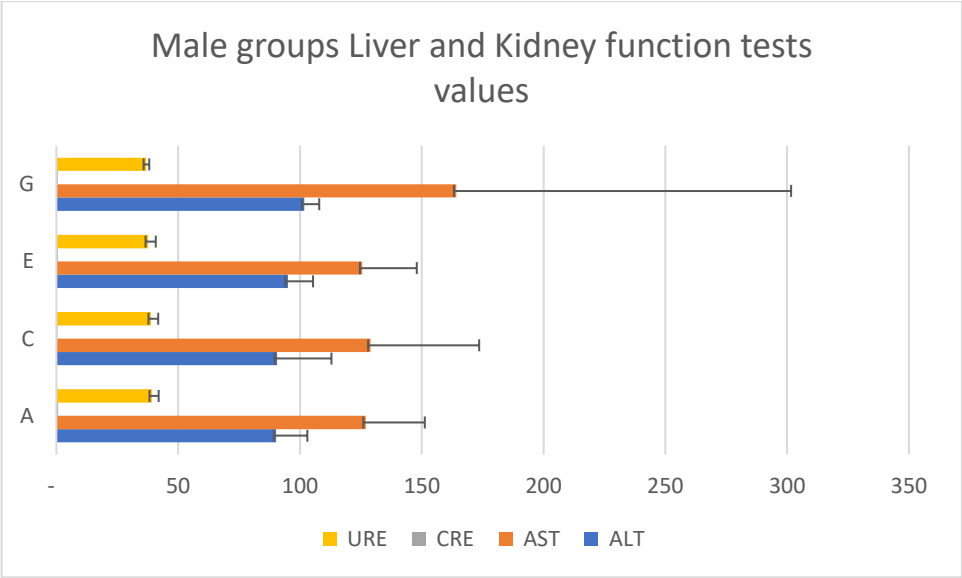



**Figure 32.** Liver and kidney function test values for male groups. A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

Even there is no statistically significance when compared based on gender; AST values exhibited an increase in "G - Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)" group. However, this effect was not observed in ALT values. Elevated levels of aspartate aminotransferase (AST) alongside relatively consistent alanine aminotransferase (ALT) levels can provide important insights into potential health conditions, especially those affecting the heart and skeletal muscles.

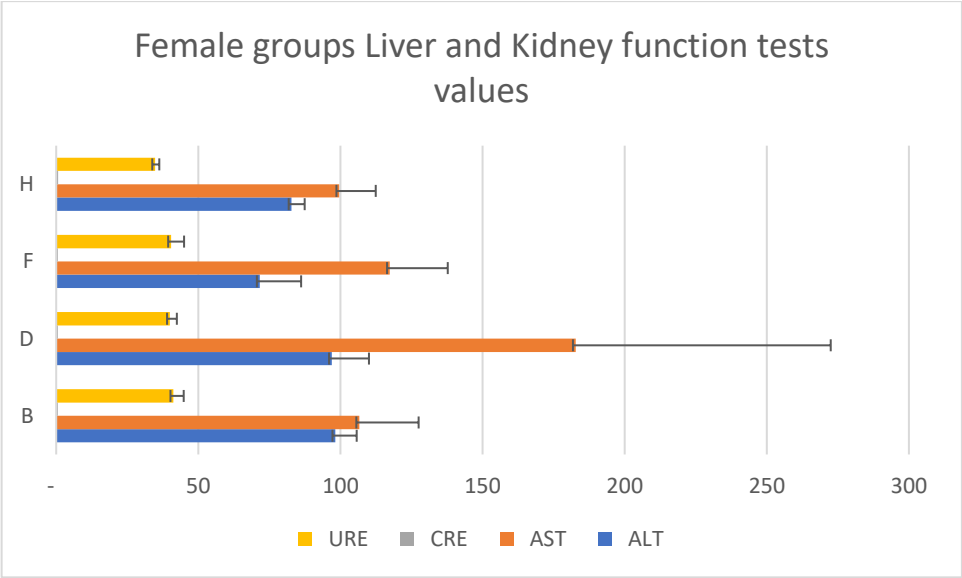



**Figure 33.** Liver and kidney function test values for female groups. A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

Even there is no statistically significance when compared based on gender; AST values exhibited an increase in the "D - Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d)" group. However, this effect was not observed in ALT values. Elevated levels of aspartate aminotransferase (AST) alongside relatively consistent alanine aminotransferase (ALT) levels can provide important insights into potential health conditions, especially those affecting the heart and skeletal muscles.

### 4.4. Plasma MDA Levels, Antioxidant Enzyme Activities (Plasma GSH-Px) Values of Rats

Table 18 illustrates the plasma MDA levels, antioxidant enzyme activities (Plasma GSH-Px) values of rat blood samples taken on the final days of the experimental procedure and respectively shown under Figures 34,35 and 36.

**Table 18.** Plasma MDA levels and Antioxidant Enzyme activities (Plasma GSH-Px) in the experimental groups' rats.

| Groups | [MDA] (nmol/g protein) | [GSH-Px] U / g protein |
|---|---|---|
| **A** | 0,07 ± 0,09 | 1621 ± 392 |
| **B** | 0,10 ± 0,08 | 1490 ± 323 |
| **C** | 0,21 ± 0,11 | 1555 ± 288 |
| **D** | 0,32 ± 0,28 | 1550 ± 590 |
| **E** | 0,25 ± 0,10 | 1439 ± 478 |
| **F** | 0,34 ± 0,19 | 2252 ± 402 |
| **G*** | 0,17 ± 0,02 | 1775 ± 436 |
| **H** | 0,34 ± 0,40 | 1243 ± 476 |

MDA, Malondialdehyde; CAT, Catalase; GSH-Px, Glutathione Peroxidase. The data is displayed as the mean plus or minus the standard deviation (mean ± SD).

*Group G Animal No 3 not used for MDA calculations. Group G Animal No 2&3 not used for GSH-Px calculations

A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

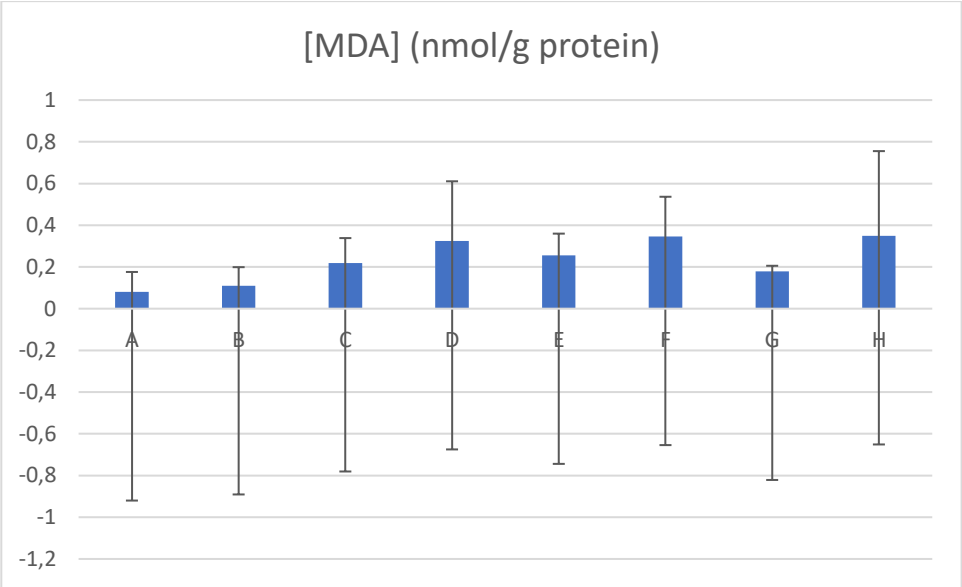



**Figure 34.** MDA levels of each experimental group. A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

The MDA (malondialdehyde) levels in the group receiving a high dose of processed (debittered) lupine seeds suspension were observed to be the lowest, although this distinction did not reach statistical significance. The results of the remaining groups showed similarity, with no significant differences detected.

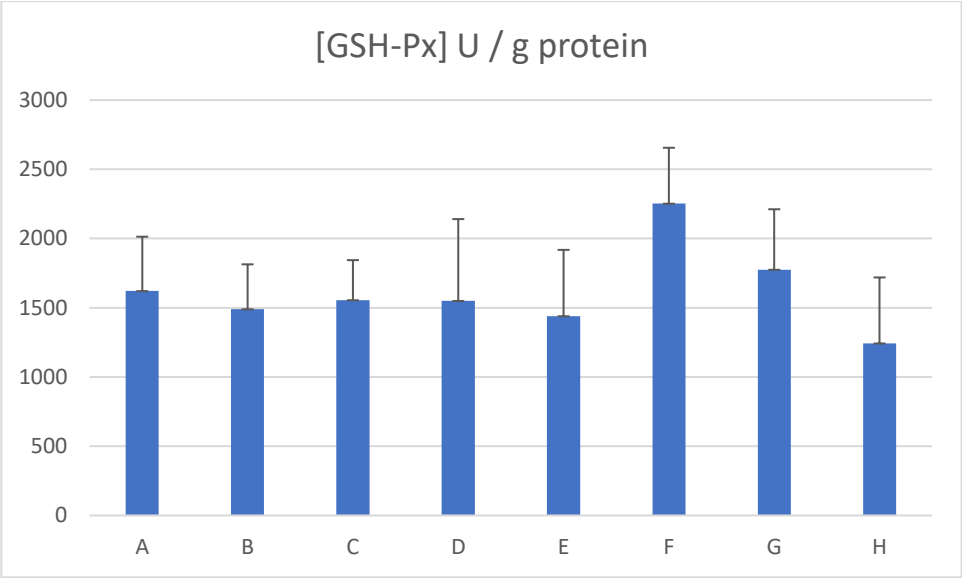



**Figure 35.** Plasma GSH-Px activities of each experimental group. A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

Additionally, while not achieving statistical significance, the group administered a low dose of processed (debittered) lupine seeds suspension displayed the highest GSH-PX (glutathione peroxidase) activity, whereas the group receiving a low dose of crude (unbittered) lupine seeds suspension had the lowest activity. Results from the other groups exhibited similarity, with no significant differences observed.

## 4.5. Results of Histopathological Analysis

Comparison of damage between groups after the evaluation of histopathology results shown in the figures below for kidney and liver.

Figure 37 displays microscopic images of Kidney tissue stained with Hematoxylin Eosin. In both group A and group B, the Kidney tubules and renal corpuscles exhibit a normal appearance. The scale bar indicates measurements of 200 micrometers at x10 magnification and 100 micrometers at x20 magnification, with x10 magnification on the left and x20 magnification on the right.

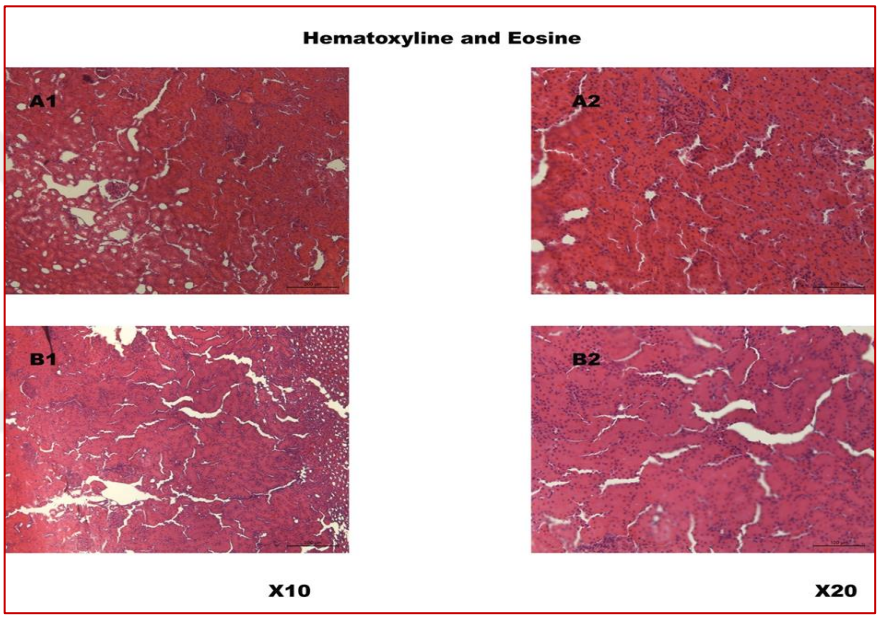



**Figure 36.** Microscopic images of kidney (Groups A and B) A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d)

Figure 38 displays microscopic images of Kidney tissue stained with Hematoxylin Eosin. In both group C and group D, the images show the presence of Capillary Congestion (indicated by white arrowheads), infiltration (indicated by black arrowheads), and necrosis (indicated by white arrows). The scale bar indicates measurements of 200 micrometers at x10 magnification on the left and 100 micrometers at x20 magnification on the right.

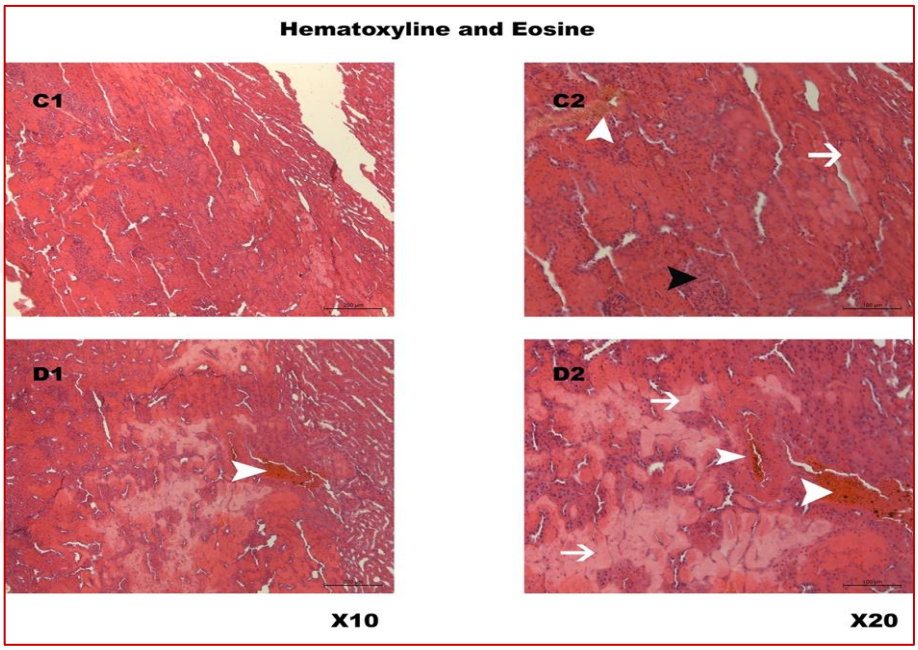



**Figure 37.** Microscopic images of the kidney (Group C&D) C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d)

Figure 39 displays microscopic images of Kidney tissue stained with Hematoxylin Eosin. In both group E and group F, the Kidney tubules and renal corpuscles exhibit a normal appearance. The scale bar indicates measurements of 200 micrometers at x10 magnification on the left and 100 micrometers at x20 magnification on the right.

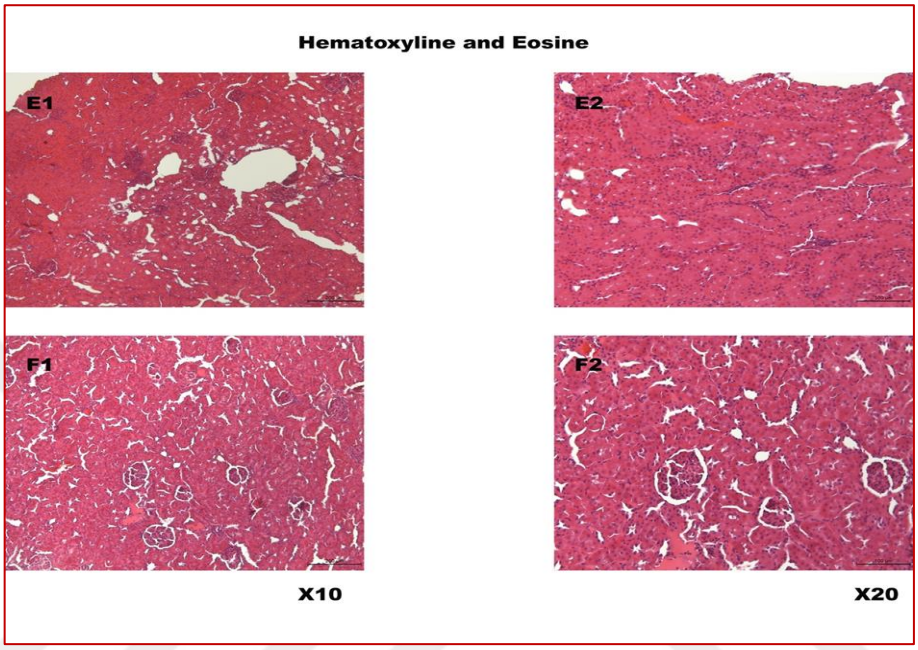



**Figure 38.** Microscopic images of the kidney (Group E&F) E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d)

Figure 40 displays microscopic images of Kidney tissue stained with Hematoxylin Eosin. In both group G and group H, the images reveal the presence of Capillary Congestion (indicated by white arrowheads), infiltration (indicated by black arrowheads), and necrosis (indicated by white arrows). The scale bar indicates measurements of 200 micrometers at x10 magnification on the left and 100 micrometers at x20 magnification on the right.

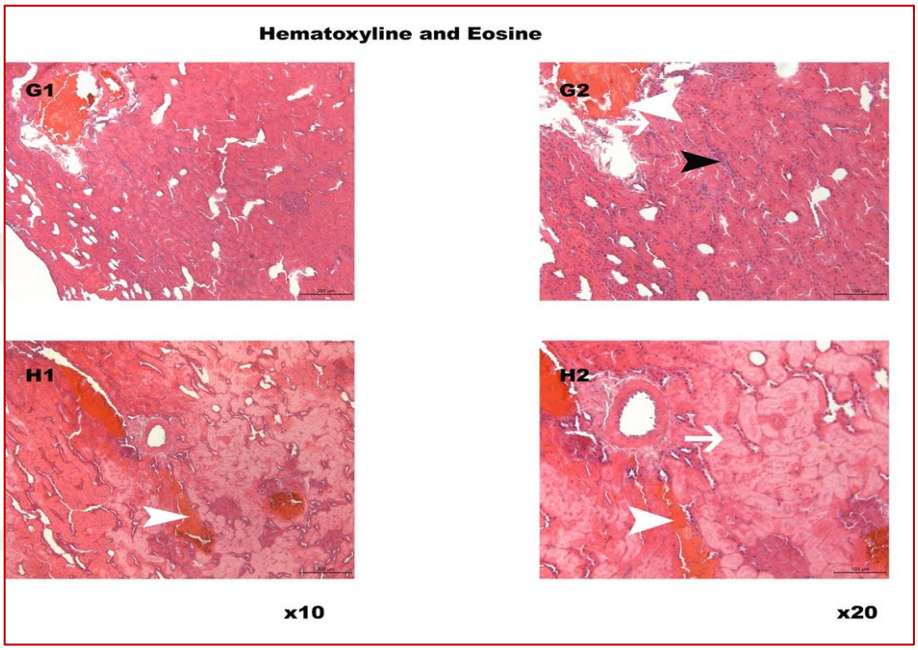



**Figure 39.** Microscopic images of the kidney (Group G&H) G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

Figure 41 displays microscopic images of Liver tissue stained with Hematoxylin Eosin. In both group A and group B, the central veins, portal triads, and hepatocytes exhibit a normal appearance. The scale bar indicates measurements of 200 micrometers at x10 magnification on the left and 100 micrometers at x20 magnification on the right.

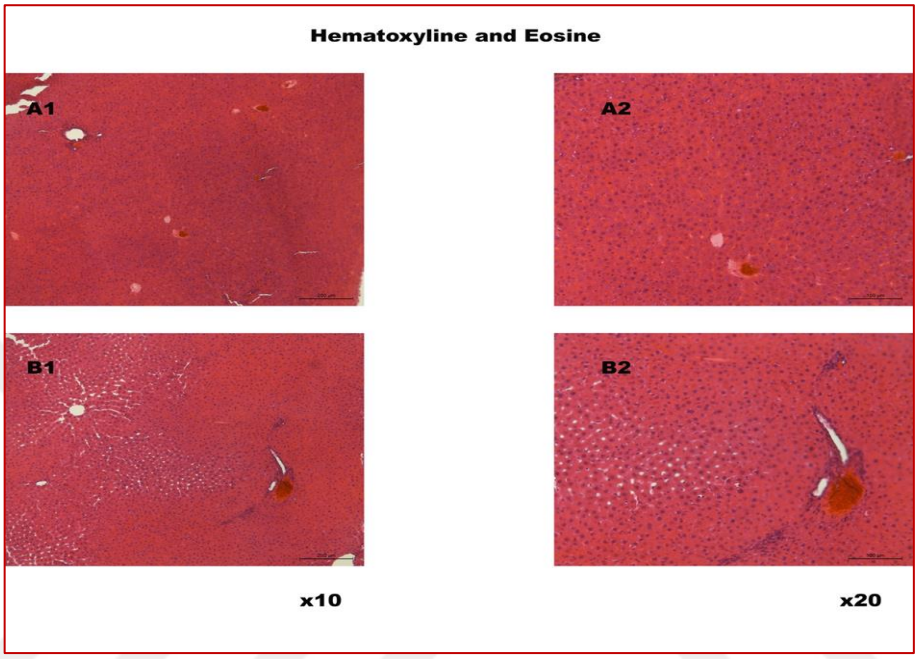



**Figure 40.** Microscopic images of the liver (Group A&B) A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d)

Figure 42 displays microscopic images of Liver tissue stained with Hematoxylin Eosin. In both group C and group D, the images show the presence of Capillary Congestion (indicated by white arrowheads), infiltration (indicated by black arrowheads), and necrosis (indicated by white arrows). The scale bar indicates measurements of 200 micrometers at x10 magnification on the left and 100 micrometers at x20 magnification on the right.

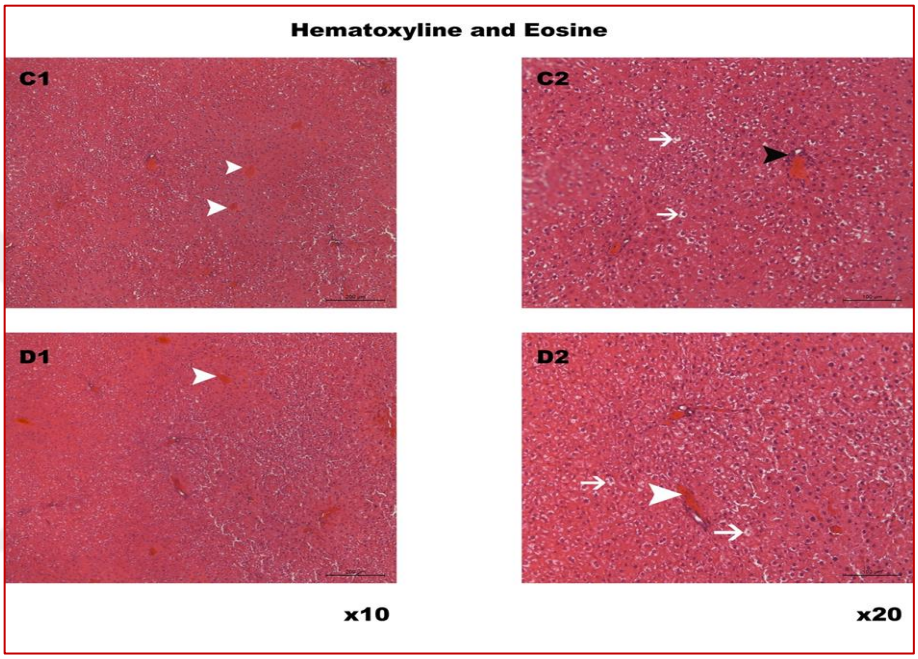



**Figure 41.** Microscopic images of the liver (Group C&D) C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d)

Figure 43 displays microscopic images of Liver tissue stained with Hematoxylin Eosin. In both group E and group F, the central veins, portal triads, and hepatocytes exhibit a normal appearance. The scale bar indicates measurements of 200 micrometers at x10 magnification on the left and 100 micrometers at x20 magnification on the right.

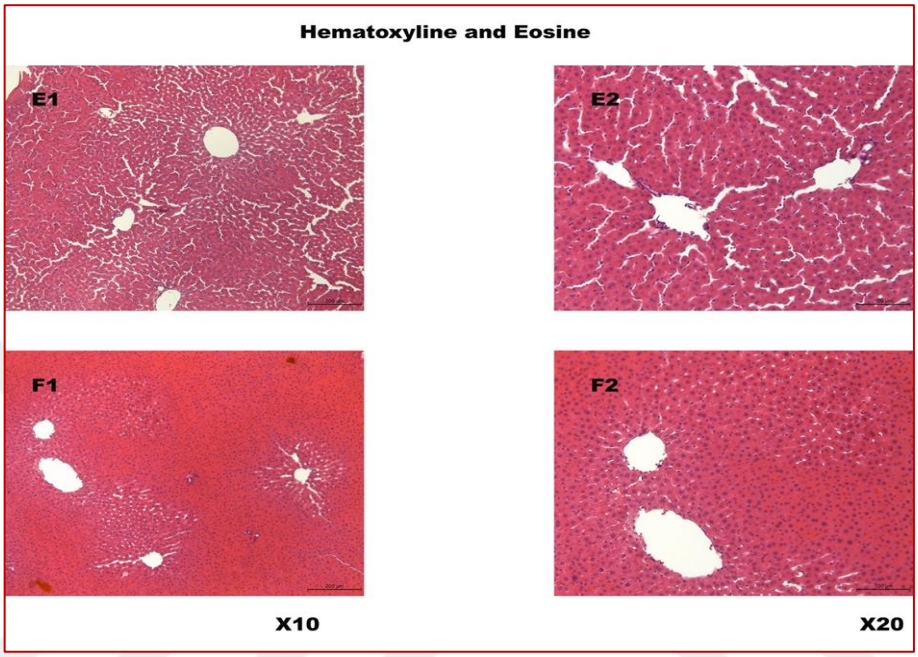



**Figure 42.** Microscopic images of the liver (Group E&F) E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d)

Figure 44 displays microscopic images of Liver tissue stained with Hematoxylin Eosin. White arrowheads point to Capillary Congestion and white arrows indicate necrosis. Necrosis and congestion were observed in both group G and group H. The scale bar indicates a measurement of 200 micrometers at x10 magnification and 100 micrometers at x20 magnification, with the x10 magnification on the left and x20 on the right.

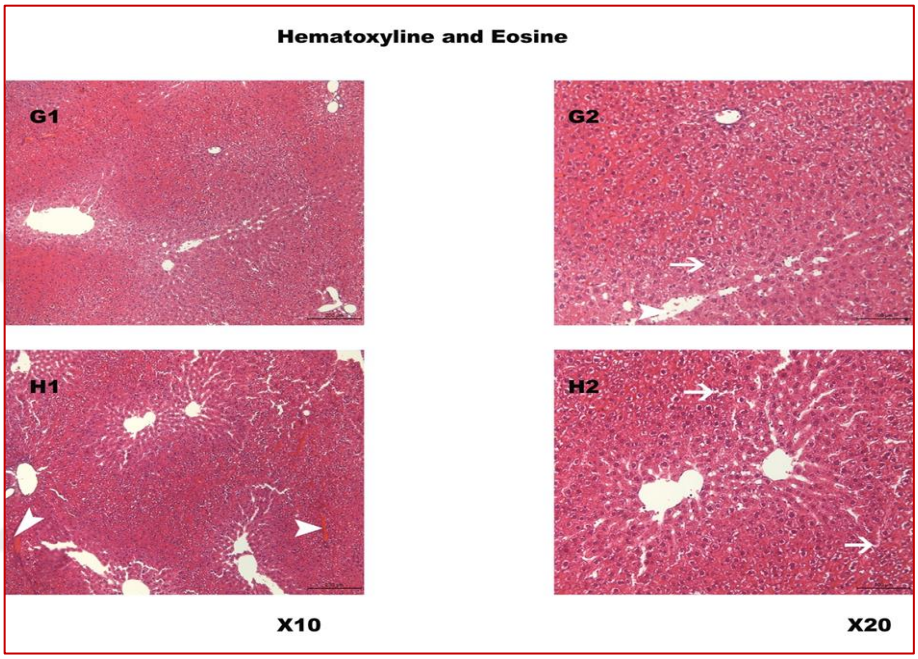



**Figure 43**. Microscopic images of the liver (Group G&H) G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

The results indicate that the consumption of substantial quantities of lupine resulted in necrosis, infiltration, and congestion at both low and high doses. However, it's noteworthy that the Kidney and liver appeared normal at both low and high doses of lupine treatment.

Table 19 shows the histopathological scores reflecting liver damage and the comparison of the total scores for liver damage shown in Figure 45 below.

**Table 19.** The histopathological scores reflecting liver damage.

| Groups | Interstitial edema (mean (min-max)) | Sinusoid capillary congestion (mean (min-max)) | Hepatocellular necrosis (mean (min-max)) | Vacuolization in hepatocytes (mean (min-max)) | Leukocyte Infiltration (mean (min-max)) | Total Score (mean (min-max)) |
|---|---|---|---|---|---|---|
| **A** | 0,60 (0 – 1) | 0,80 (0 – 1) | 0,40 (0 – 1) | 0,20 (0 – 1) | 0,80 (0 – 1) | 2,80 (2 – 5) |
| **B** | 0,80 (0 – 1) | 0,60 (0 – 1) | 0,6 (0 – 1) | 0,40 (0 – 1) | 0,80 (0 – 1) | 3,20 (2 – 4) |
| **C** | 0,89 (1 – 3) | 0,63 (1 – 3) | 0,74 (1 – 3) | 0,48 (1 – 2) | 0,40 (1 – 2) | 1,85 (5 – 10) |
| **D** | 1,80 (1 – 3) | 2,00 (1 – 3) | 2,00 (1 – 3) | 2,00 (1 – 3) | 1,40 (1 – 2) | 9,20 (5 – 10) |
| **E** | 0,20 (0 – 1) | 0,20 (0 – 1) | 0,20 (0 – 1) | 0,20 (0 – 1) | 0,40 (0 – 1) | 1,20 (0 – 2) |
| **F** | 0,20 (0 – 1) | 0,80 (0 – 1) | 0,40 (0 – 1) | 0,40 (0 – 1) | 0,20 (0 – 1) | 2,00 (1 – 3) |
| **G** | 2,60 (2 – 3) | 2,40 (2 – 3) | 2,40 (2 – 3) | 2,20 (1 – 3) | 1,80 (1 – 3) | 11,40 (10 – 14) |
| **H** | 2 (1 – 3) | 2 (1 – 3) | 2 (1 – 3) | 1,6 (1 – 2) | 2,6 (2 – 3) | 10,2 (6 – 12) |

The data is displayed as the mean plus or minus the standard deviation (mean ± SD).

A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

Upon examining the damage level ratios of liver samples, it was observed that the groups administered unbittered lupine Seeds suspension (C, D, G, and H) exhibited higher damage levels compared to the groups receiving debittered lupine Seeds suspension (A, B, E, and F).

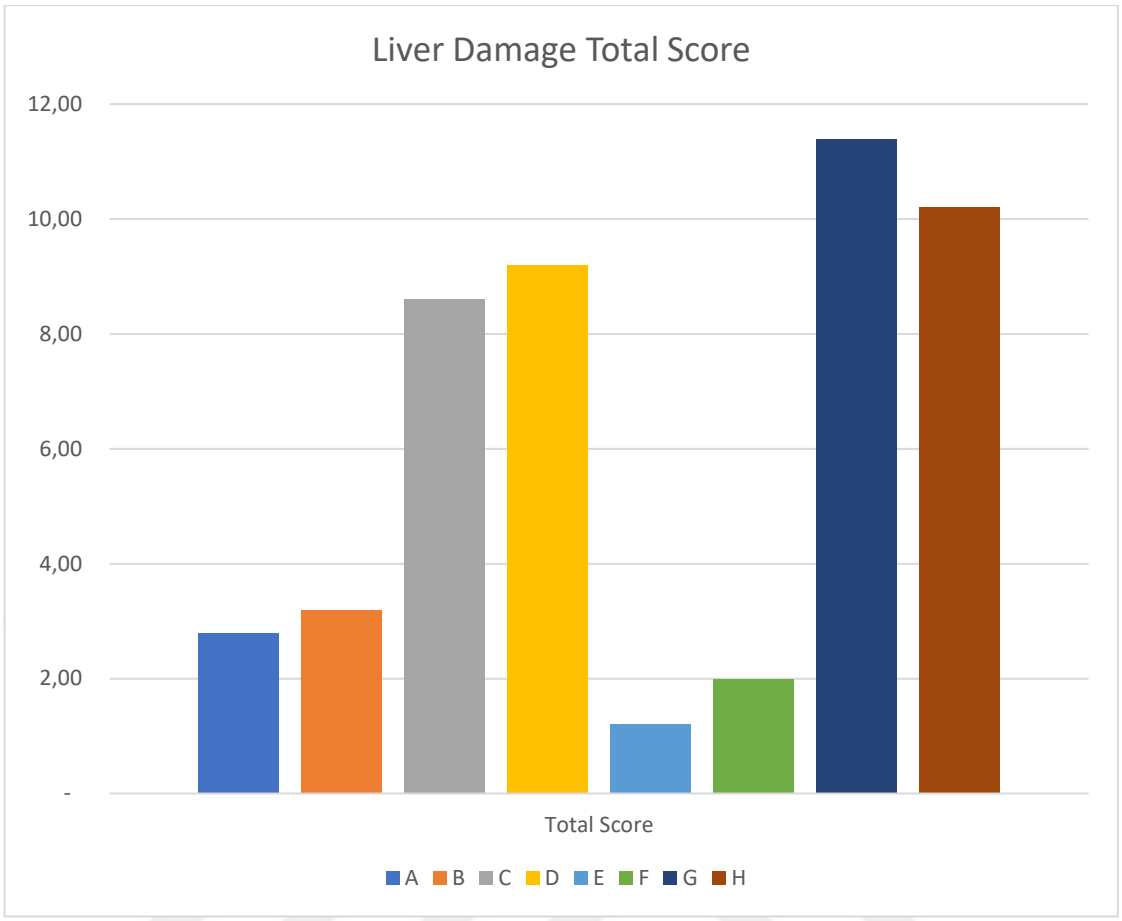



**Figure 44.** Comparison of the total scores for liver damage. A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

Upon examining the damage level ratios of liver samples, it was observed that the groups administered unbittered lupine seeds suspension (C, D, G, and H) exhibited higher damage levels compared to the groups receiving debittered lupine seeds suspension (A, B, E, and F).

Table 20 shows the histopathological scores reflecting kidney damage and the comparison of the total scores for kidney damage shown in Figure 46 below.

**Table 20.** The histopathological scores reflecting kidney damage.

| Groups | Interstitial edema (mean (min-max)) | Epithelial Damage (mean (min-max)) | Tubular Damage (mean (min-max)) | Capillary Congestion (mean (min-max)) | Mononuclear Cell Infiltration (mean (min-max)) | Total Score (mean (min-max)) |
|---|---|---|---|---|---|---|
| **A** | 0,20 (0 – 1) | 0,40 (0 – 1) | 0,20 (0 – 1) | 0,40 (0 – 1) | 0,60 (0 – 1) | 1,80 (1 – 3) |
| **B** | 0,60 (0 – 1) | 0,20 (0 – 1) | 0,20 (0 – 1) | 0,20 (0 – 1) | 0,40 (0 – 1) | 1,60 (2 – 3) |
| **C** | 0,40(1 – 2) | 0,48 (1 – 2) | 0,48 (1 – 2) | 0,48 (1 – 2) | 0,43 (1 – 2) | 1,01 (5 – 8) |
| **D** | 1,60 (1 – 2) | 1,40 (1 – 2) | 1,40 (1 – 2) | 1,20 (1 – 2) | 1,20 (1 – 2) | 6,80 (5 – 10) |
| **E** | 0,40 (0 – 1) | 0,40 (0 – 1) | 0,20 (0 – 1) | 0,20 (0 – 1) | 0,40 (0 – 1) | 1,60 (2 – 4) |
| **F** | 0,20 (0 – 1) | 0,40 (0 – 1) | 0,20 (0 – 1) | 0,20 (0 – 1) | 0,40 (0 – 1) | 1,40 (1 – 2) |
| **G** | 2,20 (2 – 3) | 1,80 (1 – 2) | 1,40 (1 – 2) | 1,40 (1 – 2) | 1,40 (1 – 2) | 8,20 (6 – 9) |
| **H** | 1,80 (2 – 3) | 1,40 (1 – 2) | 1,40 (1 – 2) | 1,40 (1 – 2) | 1,40 (1 – 2) | 7,40 (6 – 10) |

The data is displayed as the mean plus or minus the standard deviation (mean ± SD).

A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

Upon analyzing the damage level ratios of kidney samples, it was noted that the groups administered unbittered lupine seeds suspension (C, D, G, and H) exhibited higher damage levels compared to the groups receiving debittered lupine seeds suspension (A, B, E, and F).

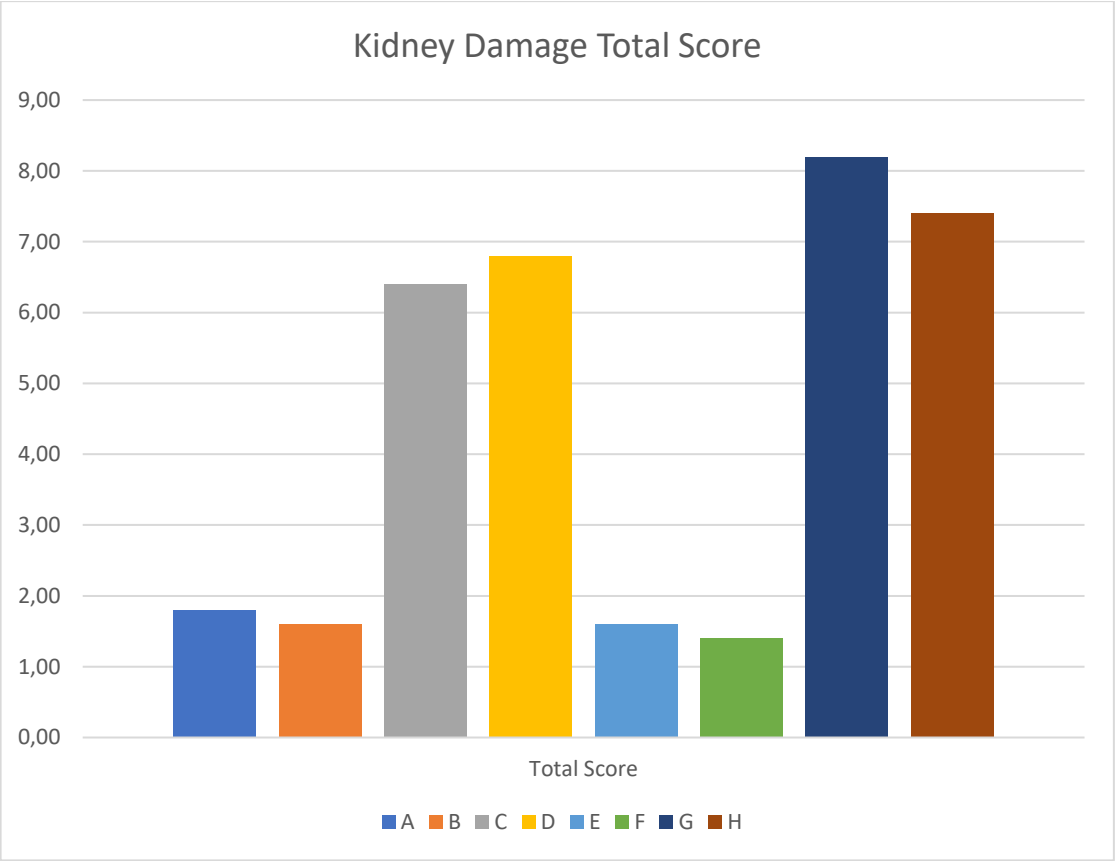



**Figure 45.** Comparison of the total scores for kidney damage. A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d): B, Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d); C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); D, Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d); E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d); F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d); G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d); H, Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

Upon analyzing the damage level ratios of kidney samples, it was noted that the groups administered unbittered lupine seeds suspension (C, D, G, and H) exhibited higher damage levels compared to the groups receiving debittered lupine seeds suspension (A, B, E, and F).

## 5. DISCUSSION

Lupine, with approximately 450 identified species, exhibits substantial variability in the composition and levels of quinolizidine alkaloids (QAs). This diversity becomes even more intricate as QA content differs not only among various genetic forms within the same species but also in response to environmental factors such as soil conditions and drought [4,5]. Among the rich array of *Lupinus* species, over 170 alkaloids belonging to the quinolizidine group have been identified, categorized based on their distinct chemical structures [5]. Tetracyclic QAs, notably sparteine, lupanine, and 13αhydroxylupanine, serve as the primary alkaloids present in the majority of *Lupinus* species [4,82]. Previous research has primarily centered on two prominent lupine alkaloids: sparteine and lupanine. It's worth noting that while both compounds exhibit a relatively low level of acute toxicity, sparteine is considered more toxic. These alkaloids have been linked to neurological issues, predominantly characterized by disruptions in motor coordination and muscle control, and reassuringly, these effects are generally reversible [96-98].

Free radicals represent species with unpaired electrons that exist independently and occupy their own electron orbits. When the unpaired electron is situated on an oxygen atom, radicals encountered located within the oxygen center, including superoxide (O2 •) and hydroxyl (OH •). These radicals possess the capacity to launch assaults on critical biomolecules such as RNA, DNA, proteins, and lipids, leading to potential damage. The detrimental consequences of free radicals extend to neuronal cell death, lipid peroxidation (LPO), DNA mutations, enzyme inactivation, and disruptions in cellular membranes. LPO is particularly associated with inflammatory processes, atherosclerosis, aging, myocardial infarction, and even cancer. The impact of free radicals on DNA manifests through mechanisms like chemical modification, depurination, and depyrimidineization of sugars and bases. Given its single-stranded nature and proximity to mitochondria, RNA is inherently more susceptible to oxidative degradation when compared to DNA [129-133, 135, 145].

Our primary aim of this research was to do a comprehensive toxicological assessment of *Lupinus albus* L. (lupine) seeds, specifically lupine seeds suspensions, to prove their safety profile. Lupine is a plant species known for its significant variability in quinolizidine alkaloid (QA) composition, which can be varied not only among different genetic forms within the same species but also in response to various environmental factors such as soil conditions and drought. The potential toxic effects of lupine seeds on

vital organs, particularly the liver and kidneys were studied, as well as their impact on oxidative stress markers in a Sprague Dawley rat model. Both debittered and crude lupine seeds were administered at different dosage levels over a 28-day period. The evaluation of organ damage, oxidative stress parameters, and other relevant factors were carried out to provide a comprehensive understanding of lupine toxicity and its implications for food safety and animal health.

Upon the conclusion of the 28-day treatment period, an extensive analysis of blood biochemistry conducted for each animal. Additionally, the rats were euthanized and proceeded to collect blood samples, liver tissues, and kidney tissues, all of which were meticulously preserved at -80 $^{o}$C to facilitate subsequent histopathological examination. To further assess the animals' well-being and the potential impact of the treatment, hemogram tests were conducted at the conclusion of the study and monitored the animals' weight gain on a weekly basis.

To distinguish between the various experimental groups, each group was designated with a specific letter and treatment, with males and females considered separately:

- A - Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d)
- B - Female processed (debittered) lupine seeds suspension (2000 mg/kg bw/d)
- C - Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d)
- D - Female crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d)
- E - Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d)
- F - Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d)
- G - Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)
- H - Female crude (unbittered) lupine seeds suspension (665 mg/kg bw/d)

Through this systematic and comprehensive approach, aim was to provide valuable insights into the safety and potential effects of Termiye consumption on key biological parameters.

Following the comprehensive evaluation of histopathological results, a clear pattern of damage emerged in both the liver and kidneys across various experimental groups. This damage assessment has provided valuable insights into the effects of lupine seeds suspension consumption, particularly when comparing crude (unbittered) lupine Seeds suspension with processed (debittered) lupine Seeds suspension, as well as the impact of different dosage levels and gender.

For the kidney, it is noteworthy that in groups A, B, E, and F, which received processed (debittered) lupine seeds suspension, the Kidney tubules and renal corpuscles appeared normal. This suggests that the consumption of debittered lupine seeds suspension at both low and high doses did not result in observable kidney damage for both males and females. In contrast, groups C and D, which received crude (unbittered) lupine seeds suspension at both dosage levels, exhibited necrosis, infiltration, and congestion in their kidney tissues. Similarly, groups G and H, also exposed to crude (unbittered) lupine seeds suspension, displayed necrosis and congestion in their kidneys. These findings imply that crude lupine seeds suspension, regardless of dosage and gender, induced necrosis and congestion in the kidneys, with infiltration being more prominent at higher doses.

In the case of the liver, groups A, B, E, and F, receiving processed (debittered) lupine seeds suspension, demonstrated normal central veins, portal triads, and hepatocytes. This suggests that the liver tissues in these groups remained unaffected by the consumption of processed lupine seeds suspension, irrespective of dosage or gender. Conversely, groups C and D, exposed to crude (unbittered) lupine seeds suspension, exhibited necrosis, infiltration, and congestion in their liver tissues. Similar liver damage, characterized by necrosis and congestion, was observed in groups G and H, also processed with crude (unbittered) lupine seeds suspension. These findings indicate that crude lupine seeds suspension, regardless of gender and dosage, led to necrosis and congestion in the liver tissues, with infiltration observed at higher doses.

In summary, the data suggest that crude (unbittered) lupine seeds suspension led to significant liver and kidney damage, as evidenced by necrosis, congestion, and infiltration. These effects were consistent across different dosages and genders. On the other hand, the consumption of processed (debittered) lupine seeds suspension did not result in observable liver or kidney damage, affirming its safety profile.

To determine statistical significance and conduct precise comparisons, various tests were employed. However, it is important to note that there were specific limitations in the availability of data for some parameters, such as Urea levels in groups G and H, which affected the choice of statistical tests and comparisons.

In this study, various statistical tests used to assess the differences in measurements among different groups. For normally distributed data with homogeneous variances, ANOVA (Analysis of Variance) utilized for comparing variables such as

Animal Weight. In cases where variances were not homogeneously distributed, specifically for RBC (Red Blood Cell) and HCT (Hematocrit) levels, opted for Welch ANOVA. For measurements that did not conform to a normal distribution, the Kruskal-Wallis test was employed.

Our findings indicate statistically significant differences in mean measurements for animal weight, ALT (Alanine Aminotransferase) levels, and WBC (White Blood Cell) levels among the groups, with a significance level of $p<0.05$. To pinpoint which groups exhibited these differences, multiple comparisons were conducted for measurements that showed significance following the ANOVA test using the Tukey test. Similarly, for measurements deemed significant after the Kruskal-Wallis test, multiple comparisons were performed using the Mann-Whitney U test. Furthermore, to control for multiple comparisons, the Bonferroni correction was applied to the p-values.

Animal Weight Comparison; our findings indicate that lupine consumption had a significant impact on animal weight. There is a significant difference between A, Male processed (debittered) lupine seeds suspension (2000 mg/kg bw/d) and C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d), with a p-value less than 0.05. The average weight of Group A is higher than that of Group C. These differences in weight suggest that lupine consumption may affect the growth and development of animals.

Furthermore, there is a statistically significant difference between G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d) and C, Male crude (unbittered) lupine seeds suspension (2000 mg/kg bw/d), with a p-value below 0.05. The average weight of Group G exceeds that of Group C. These observations suggest a complex interplay between lupine consumption and animal weight, potentially influenced by factors such as dose and duration of exposure.

ALT Level Comparison; our results indicate that the alanine aminotransferase (ALT) levels in the blood varied among different groups. There is a significant difference for ALT between G, Male crude (unbittered) lupine seeds suspension (665 mg/kg bw/d) and F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d), with a p-value less than 0.05. The average ALT of Group G is higher than that of Group F. This finding suggests that lupine consumption may have differential effects on ALT levels, possibly related to by debittering, removing the harmful compounds.

WBC Count; our results indicate that white blood cell (WBC) levels in the blood varied among different groups. There is a significant difference between E, Male processed (debittered) lupine seeds suspension (665 mg/kg bw/d) and F, Female processed (debittered) lupine seeds suspension (665 mg/kg bw/d), with a p-value less than 0.05. The average WBC of Group E is higher than that of Group F. This finding suggests that lupine consumption may influence WBC count differently in males and females. An increase in WBC count can be indicative of an immune response to infection or inflammation.

MDA Level; while not statistically significant, a notable trend was observed in malondialdehyde (MDA) levels. The group receiving a high dose of processed lupine seeds suspension displayed the lowest MDA levels, potentially indicating a reduction in oxidative stress at higher doses. This observation suggests that lupine may have a dose-dependent effect on oxidative stress markers.

GSH-PX Activity; similarly, the group receiving a low dose of processed lupine Seeds suspension displayed the highest glutathione peroxidase (GSH-PX) activity, albeit not statistically significant. This may indicate a better capacity to protect against oxidative stress at lower doses.

## 6. CONCLUSION

In conclusion, our findings suggest that the consumption of crude (non-debittered) lupine seeds led to necrosis and congestion in both the liver and kidneys, regardless of gender, at both low and high doses. The primary variation in the severity of damage was observed in the infiltration in both organs, with infiltration noted at higher doses. However, the consumption of processed (debittered) lupine seeds at both low and high doses did not negatively impact the normal functioning of the liver and kidneys, irrespective of gender. There was a statistically significant difference in body weight suggesting that lupine consumption may affect the growth and development of animals. Furthermore, our finding suggests that lupine consumption may have differential effects on ALT levels and may influence WBC count differently in males and females. Additionally, our observation suggests that lupine may have a dose-dependent effect on oxidative stress markers. On the other hand, negative results were prevented by the traditional debittering methods when correctly applied. These findings provide valuable insights into lupine toxicity and the potential benefits of debittering in reducing adverse effects on vital organs. Further research is warranted to elucidate the underlying mechanisms of lupine toxicity and its safe use.

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**APPENDIX - 1**

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

**ETİK KURUL KARARI**

| Protokol No | Toplantı Tarihi | Toplantı Sayısı | Karar No | Proje Yürütücüsü |
|---|---|---|---|---|
| 2021-052 | 14.10.2021 | 2021/10 | 2021/10-3 | Prof. Dr. Ahmet Aydın |
| 'Ham ve İşlenmiş Termiye(Lüpen=Lupinus Albus L.) Bitkisinin Toksikolojik Olarak Değerlendirilmesi' isimli proje oy birliğiyle etik açıdan uygun görülmüştür. | | | | |

| Hayvan Türü / Irkı | Toplam Hayvan Sayısı | Hayvanın Cinsiyeti |
|---|---|---|
| Sıçan / Spraque Dawley | 40 | Erkek ve Dişi |

| Görevi | Adı Soyadı | Katılım Durumu |
|---|---|---|
| Başkan | Prof. Dr. Bayram YILMAZ | |
| Başkan Vekili | Prof. Dr. Erdem YEŞİLADA | |
| Üye | Dr. Veteriner Hekim Engin SÜMER | |
| Üye | Prof. Dr. M. Ece GENÇ | |
| Üye | Prof. Dr. Rukset ATTAR | |
| 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 | |

**APPENDIX - 2**

# MERT GUNAY

## GLOBAL PROJECT MANAGER

## PROFILE

I am a results-driven pharmaceutical professional with a background in pharmacy, toxicology, and project management. My expertise includes sales and operations planning, formulation development, and change management. I am skilled in managing complex projects, working with stakeholders and cross-functional teams, and ensuring compliance with regulatory standards. With strong analytical, problem-solving, and communication skills, I am dedicated to contributing to the success of a dynamic and innovative organization in the pharmaceutical industry.

## EXPERIENCE

**Bayer I 2023 – Present**
**PS Nutritional Health Project Manager**
Lead cross functional Project Teams and are fully accountable for ensuring timely and on budget delivery of Category related projects.

**Sanofi I 2021 – 2023**
**S&OP Expert**
Manage S&OP process and analysis including demand forecasting, planning, pipeline control and within country collaboration with all stakeholders prepare system readiness for new campaigns, new launches.

**Sanofi I 2020 – 2021**
**R&D Project Management Expert**
Managing and coordinating the project team, evaluating and sharing the risks that may arise regarding time plan, unit cost and budget, having full responsibility for the new launch activities including project plan, goals, offering alternative plans, reports and updates.

**Onko Koçsel I 2018 – 2020**
**R&D Formulation Development Expert**
Formulation development for mainly oncological solid and sterile liquid form medicines, conducting validation studies with stability processes, preparing CTD formulation related parts and delivering developed products do first launches..

## EXPERTISE

- Project management
- Change management
- Sales and operations planning
- Product development
- Analytical and problem-solving
- Cross-functional team management
- Data analysis
- Communication

## REFERENCES

## EDUCATION

**2017 – Present**
**Yeditepe University**
PhD, Pharmaceutical Toxicology

**2010 – 2017**
**Yeditepe University**
Bachelor's degree, Pharmacy