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INVESTIGATION OF AMELIORATIVE EFFECTS OF 1,8 CINEOLE ON LIVER TOXICITY INDUCED BY AROCLOR 1254 IN RAT MODEL

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SIÇANLARDA AROKLOR 1254 İLE İNDÜKLENEN KARACİĞER TOKSİSİTESİNDE 1,8 SİNEOL'UN İYİLEŞTİRİCİ ETKİLERİNİN ARAŞTIRILMASI

TIBBİ FARMAKOLOJİ ANABİLİM DALI
TIBBİ FARMAKOLOJİ YÜKSEK LİSANS
YÜKSEK LİSANS TEZİ

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Scientific ethics and academic rules were carefully followed in the design, preparation, execution, research and analysis of the findings of this thesis; I declare that the findings, data and materials that are not the direct primary product of this study are cited following scientific ethics and are attributed to the cited studies.

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ÖZET

SIÇANLARDA AROKLOR 1254 İLE İNDÜKLENEN KARACİĞER TOKSİSİTESİNDE 1,8 SİNEOL'UN İYİLEŞTİRİCİ ETKİLERİNİN ARAŞTIRILMASI

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Bu çalışmada 1,8 Sineol'un erkek sıçanlarda Aroklor 1254 (A1254) ile indüklenen karaciğer toksisitene karşı koruyucu etkisinin gösterilmesi ve etki mekanizmalarının aydınlatılması amaçlandı. Poliklorlu bifeniller (PCB) dünya çapında ticari ve yerel olarak kullanılan kalıcı organik kirleticilerden (KOK) biridir. PCB'ler arasında A1254 karaciğerde, beyinde, testislerde, yumurtalıklarda ve akciğerler gibi birçok organda ciddi hasara neden olabilen bir toksik bir maddedir. Bir monoterpen olan 1,8 Sineol antimikrobiyal, antiviral, antikanser ve antioksidan etki gibi önemli farmakolojik aktivitelere sahip olduğu bilinmektedir. Bu çalışmada 32 sağlıklı genç erkek Wistar-albino sıçanlar (200–300 g) kullanıldı. Hayvanlar Kontrol, Aroklor, Sineol ve Aroklor + Sineol olarak rastgele dört eşit gruba dağıtıldı (n=8). A1254 (1 mg/kg) veya 1,8 Sineol (100 mg/kg) dozlarında mısır yağı içerisinde 30 gün boyunca sıçanlara oral gavaj ile uygulandı. Deneysel süreç tamamlandıktan sonra anestezi altında hayvanların karaciğer dokuları ve serumları alındı. A1254 ile indüklenmiş karaciğer hasarında 1,8 Sineol'un koruyucu etkisi; ELISA, RT-PCR, histopatolojik analiz ve immünhistokimyasal değerlendirme yöntemleri ile gösterildi. A1254 (1 mg/kg) verilen karaciğer dokularında total oksidan seviyesi (TOS) düzeyinin arttığı ve total antioksidan seviyesi (TAS) düzeyinin ise azalarak oksidatif stresi tetiklediğini gösterdi. Bununla birlikte; 1,8 Sineole uygulamasının TOS düzeylerini anlamlı düzeyde azalttığı, TAS düzeyini ise anlamlı düzeyde artırdığı saptandı ($p<0.05$). A1254 karaciğer dokularında pro-apoptotik Bax gen ekspresyonunu artırdı, ancak Sineol uygulaması Bax gen ekspresyonunu Araklor grubuna göre anlamlı olarak azalttı ($p<0,05$). Benzer şekilde A1254, pro-inflamatuvar sitokinler olan TNF-a, 1L-1β ve INF-ɣ gen ekspresyonlarını artırırken; Sineol uygulaması bu genlerin ekspresyonunu Araklor grubuna göre anlamlı olarak azalttı ($p<0,05$). Ayrıca 1,8 Sineole'un karaciğer dokularında A1254'ün neden olduğu oksidatif stres ve inflamasyonla ilişkili histopatolojik değişikliği önemli ölçüde azalttı ($p<0.05$). Sonuç olarak, 1,8 Sineol (100 mg/kg) sıçan karaciğer dokularında A1254 (1 mg/kg) ile indüklenen karaciğer toksisitesine karşı antiapoptotik etkisinin yanı sıra, oksidatif hasarı azaltıcı etki göstermesi, anti-inflamatuvar etkili olduğu ve histopatolojik değişiklikleri azaltması nedeni ile koruyucu potansiyel bir ajan olabilir.

Anahtar Kelimeler: 1,8 Sineol, Aroklor 1254, Karaciğer, Oksidatif Hasar, İnflamasyon

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ABSTRACT

INVESTIGATION OF AMELIORATIVE EFFECTS OF 1,8 CINEOLE ON LIVER TOXICITY INDUCED BY AROCLOR 1254 IN RAT MODEL

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In this study, the aim was to demonstrate the protective effect of 1,8 cineole against Aroclor 1254 (A1254)-induced liver toxicity in male rats and to elucidate the underlying mechanisms. Polychlorinated biphenyls (PCBs) are among the persistent organic pollutants (POPs) used globally and locally, with A1254 being a toxic substance that can cause serious damage in various organs such as the liver, brain, testes, ovaries, and lungs. 1,8 Cineole, a monoterpene, is known to possess significant pharmacological activities such as antimicrobial, antiviral, anticancer, and antioxidant effects. Thirty-two healthy young male Wistar albino rats (200–300 g) were used in this study. The animals were randomly divided into four equal groups: Control, Aroclor, Cineole, and Aroclor + Cineole (n=8). A1254 (1 mg/kg) or 1,8 cineole (100 mg/kg) was orally administered to the rats in corn oil for 30 days. After the experimental process was completed, liver tissues and sera were collected from the animals under anesthesia. The protective effect of 1,8 cineole against A1254-induced liver damage was demonstrated using ELISA, RT-PCR, histopathological analysis, and immunohistochemical evaluation methods. A1254 (1 mg/kg) administration increased the total oxidant status (TOS) level and triggered oxidative stress by decreasing total antioxidant status (TAS) in liver tissues. However, 1,8 cineole application significantly reduced TOS levels and increased TAS levels ($p<0.05$). A1254 increased the pro-apoptotic Bax gene expression in liver tissues, while cineole application significantly decreased Bax gene expression compared to the Aroclor group ($p<0.05$). Similarly, A1254 increased the gene expressions of pro-inflammatory cytokines TNF-α, IL-1β, and INF-γ in liver tissues, whereas cineole application significantly decreased the expression of these genes compared to the Aroclor group ($p<0.05$). Moreover, 1,8 cineole significantly reduced the histopathological changes associated with A1254-induced oxidative stress and inflammation in liver tissues ($p<0.05$). In conclusion, 1,8 cineole (100 mg/kg) may serve as a protective agent due to its anti-apoptotic effect, reduction of oxidative damage, anti-inflammatory efficacy, and mitigation of histopathological changes in rat liver tissues induced by A1254 (1 mg/kg).

Keywords: 1,8 Cineole, Aroclor 1254, Liver, Oxidative damage, Inflammation

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Hadiqa ARSHAD

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SYMBOLS & ABBREVIATIONS

1,8 C	1,8 Cineole
A1254	Aroclor 1254
A1260	Aroclor 1260
A1248	Aroclor 1248
Aroclor	Aroclor 1254
AChE	Acetylcholinesterase enzyme
AMPK	Adenosine 50-monophosphate activated protein kinase
AD	Alzheimer's disease
Aβ	Amyloid beta
AKBA	Acetyl-11-keto-β Boswellic Acid
ANOVA	Analysis of variance
AhR	Aryl hydrocarbon receptor
Akt	Protein kinase B
Bcl-2	B-cell lymphoma 2
Bax	Bcl-2-associated X protein
BPA	Bisphenol A
BALF	Bronchoalveolar lavage fluid
BTLAB	Bioassay technology laboratory
Cineol	1,8 Cineole
CYP	Cytochrome P450
COPD	Chronic obstructive pulmonary disease
CYP1A1	Cytochrome P450 1A1
CYP1B	Cytochrome P450 1B
CYP1A	Cytochrome P450 1A
COX-2	Cyclooxygenase-2
CAT	Chloramphenicol acetyltransferase
C57BL/6	Common inbred strain of laboratory mouse
dl-PCBs	Doxin like-Polychlorinated Biphenyls
DNA	Deoxyribonucleic acid
DMAPP	Dimethylallyl disphosphate
DAB	3,3'-diaminobenzidine
DSS	Dextran sodium sulphate
dNTP	Deoxynucleotide triphosphate
eNOS	Endothelial nitric oxide synthase
EROD	Ethoxy resorufin O-deethylase
EDTA	Ethylene diamine tetraacetic acid
ERK	Extracellular regulated kinase
FC	Fold change
FVB	An albino inbred laboratory rice
GSH	Glutathione
GST	Glutathione S-transferases
GGT	Gamma-glutamyl transferase
GTP	Guanosine triphosphate
GPx	Glutathione peroxidase
HSV-1	Herpes Simplex Virus 1
HDL	High-density lipoprotein
HRP	Histidine-rich protein
HE	Hematoxylin-eosin

HPTA	Hypothalamus-pituitary-Testes-Axis
HaCaT	Human epidermal keratinocytes
HUVECs	Human umbilical vein endothelial cells
H9C2	Embryonic rat cardiomyocytes
HSCs	Hematopoietic stem cell
IARC	International agency for research on Cancer
IPP	Isopentenyl disphosphate
IFN-γ	Interferon-gamma
IL-1β	Interleukin 1β
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-17A	Interleukin 17 A
IGF	Insulin-like growth factor
ISO	Isoproterenol
IRE1	Inositol-requiring enzyme type 1
Keap1	Kelch-like ECH-associated protein 1
LP	Lipid peroxidation
LPS	Lipopolysaccharides
MeSO2	Methyl suphonyl
MVA	Mevalonic pathway
MEP	Methyl erythritol phosphate pathway
MAPK	Mitogen-activated protein kinase
M6P	Mannose 6-phosphate
mTOR	Mammalian target of rapamycin
MPO	Myeloperoxide
MPT	Mytochondrial pore transition
MDA	Malondialdehyde
MROD	Methoxyresorufin O-deeathylase
mRNA	Messenger RNA
Ndl-PCBs	Non Dioxin like-Polychlorinated biphenyls
NF-κβ	Nuclear factor kappa light chain enhancer
Nrf2	Nuclear factor of erythroid 2-related factor 2
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NOQ1	Quinone oxidoreductase
NO	Nitric oxide
NLR	Node-like receptors
NLRP3	Node-like receptors family pyrin domain
OVA	Ovalubumin induced asthma
OH-PCBs	Hydroxylated-Polychlorinated biphenyls
POPs	Persistent organic pollutants
PCBs	Polychlorinated biphenyls
PCB 136	Polychlorinated biphenyl 136
PCB 95	Polychlorinated biphenyl 95
PCB 153	Polychlorinated biphenyl153
PCDFs	Polychlorinated dibenzofurans
PCDDs	Polychlorinated-p-dioxins

PROD	Pentoxyresorufin O-deethylase
PPAR	Peroxisome proliferator activated receptor
P53	Tumor protein
PI3K	Phosphatidylinositol 3-kinase
PLA2	Phospholipase A2
PBS	Phosphate buffer solution
PAMPs	Pathogens associated molecular patterns
PRPs	Pattern recognition receptors
PARP	Poly adenosine diphosphate-ribose polymerase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RT-PCR	Reverse transcriptase Polymerase chain reaction
RSV	Respiratory syncytial virus
SOD	Superoxide dismustase
STAT1	Signal transducer activator of transcription 1
STAT3	Signal transducer activator of transcription 3
SULTs	Cytosolic tranferases
SPSS	Statistical package fort the social sciences
TEQs	Toxic equivalents
TEF	Toxic equivalency factor
TGF	Transforming growth factor
TGFβ-1	Transforming growth factor beta
TREM-1	Triggered receptors expressed on myeloid cells-1
TNF-α	Tumore necrosis factor-alpha
TAS	Total antioxidant status
TOS	Total oxidant status
TCDD	2,3,7,8 tetrachloro dibenzo-p-dioxin
UDP	Uridine disphosphate
UGTs	Glucouronosyl transferases (UGTs)
UVB	Ultraviolet B
XBP1	X-box binding protein 1

1. INTRODUCTION

The liver's structure and function make it a frequent target for toxicity from xenobiotics. Hepatocytes, which flow directly to the liver via portal venous blood, are exposed to xenobiotics without systemic modification or dilution, leading to hepatotoxicity through specific chemical interaction. Although hepatocytes have a considerable detoxifying ability, exposure to high amounts of reactive oxidative species (ROS) may disturb their redox status. If repair mechanisms do not balance these ROS-mediated stress events, the damaged cells die or suffer apoptosis (Friedman et al., 1996).

Persistent Organic Pollutants (POPs) having a lipophilic identity, consist of 12 chemical substances classified as “Initial POP or Dirty Dozen”, including nine pesticides and the other three are environmental pollutants such as polychlorinated biphenyls (PCBs) (Jeong et al., 2014). PCBs as an important group of POPs are typically yellow or deep yellow with an oily liquid consistency. Due to their distinct characteristics like insulating ability, acid resistance, hydrolysis, oxidation resistance, and thermal stability, they are widely used in a range of commercial things like transformers, capacitors, paints, pesticides, adhesives, paints, and much more (Fitzgerald et al., 2007; Safe, 1994).

Furthermore, PCBs are halogenated organic compounds with 209 congeners. They are divided into two groups based on their chemical structure and hazardous properties: dioxin-like (dl-PCBs) having 12 congeners and non-dioxin-like (Ndl-PCBs) consisting of 197 congeners (Goel et al., 2016). Because of their high toxicity and large quantities in the environment, the 12 dioxin-like and 6 indicator PCBs were typically of concern among the 209 PCB congeners. Because of its widespread prevalence and possible emission sources linked with pigment yellow synthesis, 3,30-dichlorobiphenyl (PCB-11), a non-Aroclor PCB congener, has received much attention in recent years (Lehmler et al., 2010; Shang et al., 2014). Humans are exposed to PCBs in a variety of ways, including food, water, air, and soil as external routes and human milk, blood, and other bodily fluids as internal pathways. It is believed that approximately more than 90%

of different PCBs enter people's bodies through food, and alternative routes of exposure do not contribute significantly to the general population's PCB body burden ("Chemical agents and related occupations," 2012; Journal, 2005). Even though PCB manufacture has been halted since the 1980s, its widespread use over the last two decades has resulted in their continued discovery in people, wildlife, and numerous environmental media. These fears are fueled not just by the massive volumes of PCBs discharged into the environment, but also by their developmental toxicity, cancer risk, and threats to human and wildlife health. Long-term PCB exposure causes the most harm to the human skin and liver, as well as the gastrointestinal tract, immunological system, and neurological system (Di et al., 2001).

Aroclors (A) are commercially utilized PCB mixes with a four-digit number, the first two of which relate to the number of carbon atoms connected to the biphenyl ring and the last two to the proportion (by weight) of chlorine. This family's most often utilized compounds include A1260, A1248, A1242, and A1254. A1254 and PCBs exert their harmful effect by generating free radicals, inducing hepatic oxidative stress, thereby affecting the detoxification process (Murugesan et al., 2005). A1254-induced cytotoxicity, mitochondrial dysfunction in isolated rat hepatocytes, and in vitro, genotoxicity hazard assessment in rat liver and human liver subcellular fractions were all reported in one of the investigations (Obach et al., 2008).

Terpenes, also known as isoprenoids, a diverse group responsible for plant fragrance, taste, and pigmentation, include larger classes like sterols and squalene, found in animals and primarily found in plants. An isoprene unit having a chemical formula C5H8 is a hydrocarbon, serving as a building block for terpenes. Terpenes are classified according to their structure and the number of isoprene units they contain. Terpenes are gaining commercial interest due to their potential in disease prevention, treatment, natural insecticides, antimicrobial agents, agricultural storage, and synthesis of valuable compounds, as well as their potential in preventing and treating diseases like cancer (Tetali, 2019).

As a saturated monoterpene, 1,8-cineole is commonly used in flavored, cosmetic, or scent goods such as bath additives, mouthwashes, and insect repellants. 1,8 Cineole exhibits pharmacological activities by modulating the nuclear pathway, reducing the synthesis of reactive oxygen species (ROS), and promoting anti-inflammatory and antioxidant properties. 1,8-cineole's promising preclinical profile in pharmacology

research is hindered by poor stability, necessitating the development of suitable formulations for in vivo application. Also, The National Medical Products Administration in China has approved 1,8-cineole, a main compound of Eucalyptus limonene-pinene soft capsules, for the clinical treatment of pneumonia (Cai et al., 2021; Z. Jiang et al., 2019). Different studies have shown variety of essential oils possessing 1,8 Cineole as hepatoprotective agents like *Achillea biebersteinii Afan, Achillea wilhelmsii, Ajuga iva, Artemisia capillaris, Croton zehntneri, Hyptis crenata, Rosmarinus officinalis, Thymus capitatus* and *Zingiber officinale* (Daoudi & Bnouham, 2020).

1.1. Aim & Objective

This thesis aims to investigate the therapeutic effect of 1,8 Cineole against liver toxicity induced by Aroclor 1254 in rats and the possible pathways of oxidative damage, inflammation, and apoptotic cell death, which play a role in the mechanism of action.

2. GENERAL INFORMATION AND LITERATURE REVIEW

2.1. The Hepatic System

The liver is the largest organ in humans, detoxifying ingested compounds from the digestive system before their distribution into the bloodstream as the principal role. However, the liver also serves numerous additional purposes, such as the production of bile, proteins for blood, cholesterol, and specific proteins to assist the transport of fats, storing and releasing glucose, conversion of toxic ammonia to urea, and producing immunological components.

With the recent advancements and rapid development in experimental medicine, selecting animal models whose anatomy resembles human anatomy is critical during the experimental investigation and surgical procedures in the laboratory (Vdoviaková et al., 2016). Laboratory rats have organ anatomical features comparable to humans, making them ideal for anatomical, physiological, and biochemical studies on the digestive system. The most often used animal model in experimental liver and intestinal transplantation is this laboratory species (Lopes et al., 1998). The rat liver has been the subject of substantial research due to recent data demonstrating its involvement in multiple processes (e.g., secretion, proliferation, absorption, and neo-angiogenesis) crucial for its pathophysiology. The hepatic artery, portal vein, and lymphatic vessels, which create an integrated system of liver tissue, maintain the metabolism and function of the liver.

A detailed description of rat liver morphology and vasculature is required for experiments on these laboratory animals. Other researches describe the morphology, anatomical differences, and similarities between rat liver and human liver. Despite having lobes in the liver, the difference rises in the number as rat liver has four lobes named

right, left, middle, and caudate lobe, whereas, on the other hand, the human liver is divided into eight segments (Madrahimov et al., 2006; Martins & Neuhaus, 2007).

Rats' intrahepatic bile ducts are identical to human bile ducts. However, the extrahepatic bile ducts are more superficial in rats, and the common hepatic duct is longer than in humans. Secondary branches for the liver lobes have significant morphological variants. Rats lack gallbladder, unlike other laboratory animals like pigs (Martins & Neuhaus, 2007).

Furthermore, the rat hepatic system shares similarities with the human liver regarding drug metabolism. Many enzymes participating in drug metabolism, for example, the cytochrome P450 family of enzymes, are present in both species. This similarity makes rats a valuable model for studying drug metabolism and toxicity, providing insights into how drugs are processed and eliminated in humans.

It is important to note that while there are similarities between the rat hepatic system and the human liver, there are also differences. These differences include variances in enzyme expression, metabolic rates, and certain physiological aspects. Therefore, while the rat hepatic system can serve as a valuable model for understanding human liver function and drug metabolism, caution must be exercised when extrapolating findings directly from rats to humans.

The rat hepatic system shares structural and functional similarities with the human liver. This similarity makes rats a valuable model for studying liver physiology and drug metabolism. However, it is crucial to consider the species-specific differences when interpreting experimental data.

2.2. Polychlorinated Biphenyls

The link between human health and the living environment has been an increasing focus of concern as global industrialization and environmental deterioration progress rapidly. Polychlorinated biphenyls (PCBs) are a class of organohalogen chemicals having 209 potential chemical formulae (also known as congeners) containing 1 to 10 chlorine atoms (equivalent to 10 homologs). From the late 1920s to the early 1990s, about 1.3 million tons of technical PCB mixes were manufactured globally in eleven nations

presently recognized as the United States (US), Germany, Russia, France, the United Kingdom (UK), Japan, Italy, Spain, the Czech Republic, China, and Poland. However, between 1920 and 1980, roughly 1.5 million tons of legacy PCBs were manufactured. Furthermore, PCBs are still generated accidentally and can be detected in a large number of consumer items (Guo et al., 2014; Jahnke et al., 2019). Because PCBs are chemically inert and have excellent fire retardant, thermal conductivity, and electric insulation properties, they are widely used as dielectric fluids in transformers and capacitors, heat transfer fluids, hydraulic fluids, and additives in a variety of closed and open systems (INTER, 1999). Because of their lipophilicity, PCBs, like other persistent organic pollutants (POPs), accumulate in living creatures and amplify in terrestrial and marine food webs. Because of mounting environmental and human health concerns, their industrial manufacture was outlawed in the late 1970s. Recent investigations, however, show that PCBs are formed accidentally as part of some commercials as well as industrial processes, such as the creation of adhesives, pigments, plastics, etc. (Anezaki et al., 2014; Hu et al., 2010).

2.2.1. PCBs epidemiology

Epidemiological investigations have revealed that PCBs are strongly linked to the development of cardiovascular illnesses, liver diseases, hormone problems, immunological effects, neurologic impacts, and genetic damage, among other things (Crinnion, 2011; Hagmar, 2003; Yum et al., 2010).

2.2.2. Chemical properties of PCBs

PCBs are a class of synthetic aromatic compounds. They are made up of a biphenyl structure that is linked to hydrogen and chlorine atoms through the chemical formula C12HxCly, where x and y are integers ranging from 1 to 10, and x + y = 10. According to the International Union of Pure and Applied Chemistry, 209 PCB congeners differ by the amount of hydrogen atoms substituted by chlorine atoms and their location

on the biphenyl rings. This variation in the PCB molecule dictates its physical and chemical characteristics, as well as its toxicity.

The phenyl rings can rotate around the C-C bond, but as the number of chlorines in the ortho positions increases (2, 2, 6, 6), this rotation becomes more challenging. The two phenyls can align co-planarly since there are no ortho-chlorines. The aromatic system stabilizes the plane between the phenyls as the C-C bond gains greater double bond features. However, when the number of chlorine atoms in the ortho position increases, the possibility of establishing planarity diminishes. This is crucial for the toxicity of the particular congener since the toxicity of co-planar non-ortho congeners has a "dioxin-like" toxicity. Mono-ortho congeners can also be slightly co-planar, but co-planarity is impossible if there are two or more chlorines in the ortho position (ATSDR & Disease Registry. Atlanta, 2000; Othman et al., 2022).

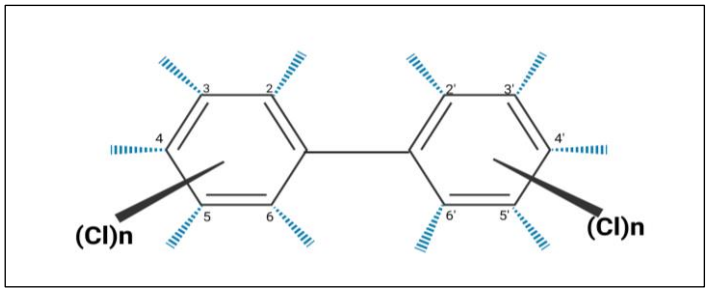

Figure 2.1. The chemical structure of the PCBs family

2.2.3. PCBs classification

PCBs have various chemical and biological characteristics depending on the position and quantity of chlorine atoms. As a result, PCBs were classified into two groups. One group is classified as "dioxin-like" due to structural and toxicity similarities with polychlorinated dibenzo-p-dioxins. The absence of chlorine atoms at ortho-positions permits the PCB to adopt a coplanar structure, which is the most evident property of this group (Song et al., 2011).

Another group is "non-dioxin-like" congeners, which have one or more chlorine atoms in ortho-positions and diminish molecule planarity. The metabolic process, on the other hand, may add a function group, such as hydroxy, in the ortho-position of a benzene

ring. An ortho position addition was discovered to enhance the dihedral angle of the resultant PCB metabolites, which not only modified the category of parent PCBs but also has substantial consequences for biological activities and toxicological profile alterations in the long run (Song et al., 2011).

2.2.4. PCBs toxicity

PCBs are one of the "dirty dozen" POPs mentioned in Annex A (elimination) and Annex C (unintentional production) of the Stockholm Convention (Melymuk et al., 2022). Here are some key reasons why concern for PCBs is warranted:

2.2.4.1. Persistence and bioaccumulation

PCBs are very persistent in the environment, which means they do not break down easily and can persist for an extended amount of time. They can also bioaccumulate in the food chain, accumulating in greater amounts as they ascend the food chain. This may culminate in elevated PCB levels in creatures at the top of the food chain, including humans (Melymuk et al., 2022).

2.2.4.2. General toxicity

PCBs are poisonous to both people and wildlife. They have been proven to affect the immunological, reproductive, endocrine, and neurological systems. Long-term PCB exposure has been correlated with developmental delays in children, an increased risk of certain malignancies, and other health consequences (Melymuk et al., 2022).

2.2.4.3. Environmental impact

PCBs have been found in air, water, and soil all across the world, even in remote places distant from their initial origins. Because of their endurance in the environment, they can accumulate and have long-term effects on ecosystems. PCBs have been related to population decreases in several kinds of aquatic creatures, particularly fish.

2.2.4.4 Remediation challenges

PCBs can be tricky to clean up after they have been introduced into the environment. They have the potential to contaminate soil, sediment, and water, necessitating difficult and costly cleanup measures. PCBs are frequently discovered in outdated electrical equipment such as transformers and capacitors, which makes appropriate disposal and safe handling difficult (Melymuk et al., 2022).

2.2.5. Toxicokinetics of PCBs

Toxicokinetics refers to the study of how a toxic substance, such as polychlorinated biphenyls (PCBs), is absorbed, distributed, metabolized, and eliminated in the body. Factors affecting the toxicokinetics of PCBs are generally initial clearance of PCBs by the liver and muscles, fat components in the body for the storage of PCBs half-life (Kania-Korwel & Lehmler, 2016). The liver and muscle are crucial for the initial dispersion of PCBs in mammals due to their strong perfusion, large mass, and high xenobiotic removal capacity. This will result in rapid initial clearance of PCBs. Initial clearance changes with different PCB congeners whereas the half-lives of PCB congeners significantly differ based on the level of chlorination and the substitution pattern. In rats, for example, PCB 136 has a tenfold shorter half-life than PCB 153 (Kania-Korwel et al., 2010; Matthews et al., 1980).

The second important factor is a fat component that fluctuates the distribution of PCBs. PCBs are stored in fat compartment due to high lipophilicity, with the highest

tissue-to-blood partition coefficient, like the human adipose tissue-to-blood partition coefficient for PCB 95 ranging between 45 and 62 (Parham et al., 1997).

2.2.6. PCBs a threat to human health

Toxicological studies have confirmed that PCBs are potentially toxic to fish, mammals, and humans, and can cause hepatotoxicity, reproductive and developmental toxicity, and neurotoxicity, among others (Berghuis et al., 2019; Guo et al., 2020; Xie et al., 2019).

The atmosphere has a significant impact on the long-term transformation and dispersion of POPs. The study of PCB contamination in the atmosphere not only helps us understand PCBs' long-range transport capacity and migration process, but it also helps us understand the possible threats presented by breathed PCBs to human health. PCB poisoning in the atmosphere has been widely observed in China during the last 15 years. Soil is an essential environmental substrate because it serves as both a source and a sink for high-chlorinated PCB homologs. PCBs in soils are mostly sourced from atmospheric deposition. Numerous studies have established the presence of PCBs in soils, as has the presence of other POPs. Higher soil PCB concentrations are frequently linked with places with a history of industrial activity, such as transformer production sites or old landfill sites. PCBs can enter water bodies from a variety of sources, including industrial discharges, runoff from contaminated soil, and incorrect disposal of PCB-containing products. Surface water, such as rivers, lakes, and coastal regions, can act as PCB reservoirs. PCBs tend to collect in sediment, where they can remain for extended periods and endanger aquatic life. Drinking water supplies may potentially be contaminated by PCBs, particularly in locations near industrial operations. PCBs can bioaccumulate and biomagnify in the food chain, resulting in larger amounts in higher trophic-level species. PCBs are known to accumulate in substantial amounts in aquatic creatures, notably fish. Due to nutritional exposure, wildlife living in polluted regions, such as birds and animals, can have high PCB concentrations. Human ingestion of polluted fish and wildlife is a major source of PCB exposure. Because of previous or present industrial operations, several areas are deemed PCB hotspots. These include regions near PCB manufacturing plants, electrical transformer installations, and hazardous waste disposal sites. Because

of their vulnerability to PCB buildup and possible implications on sensitive species and habitats, sensitive places such as wetlands, estuaries, and ecologically significant regions may also be in danger (Munawar et al., 2021).

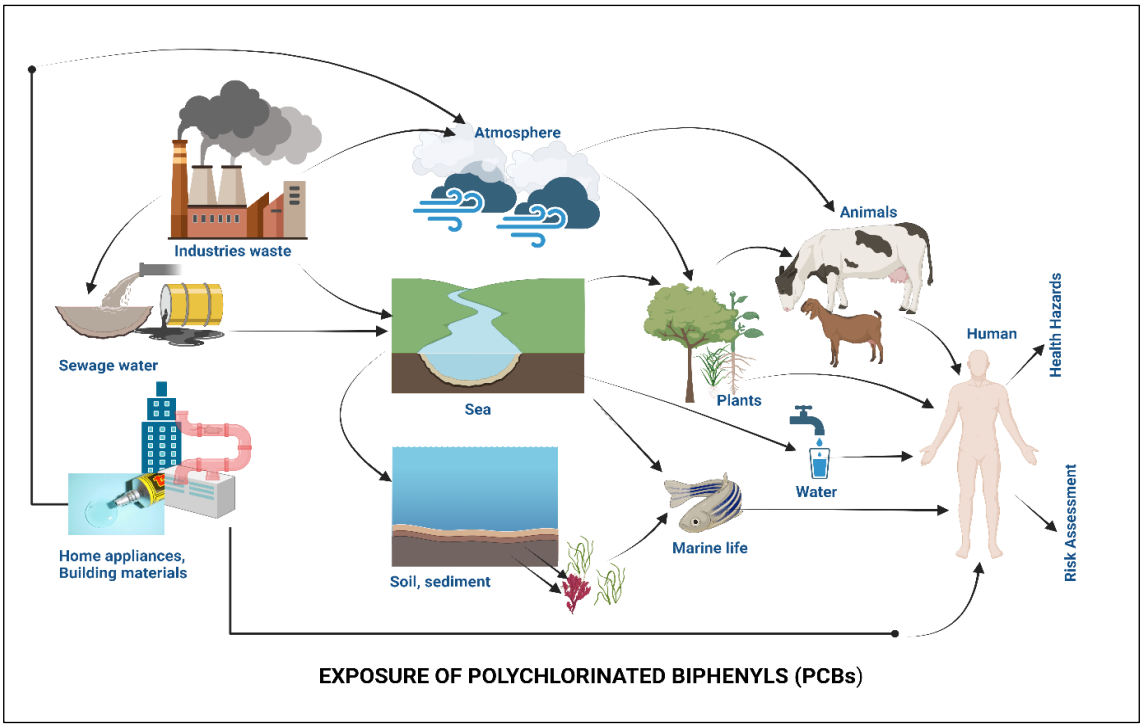

Figure 2.2. Industrial wastes, sewage water as well and extra use of PCBs in industrial and domestic materials contaminate the air and water. Humans are later exposed to PCBs by the consumption of seafood, dairy products, drinking water, air, and water. Excessive consumption can lead to serious health concerns and problems in human life.

2.2.7. PCBs risk assessment

It is difficult to regulate PCB risk assessment due to the varied concentration of PCBs in environmental matrices and their diverse mechanisms of toxicity. As a result, the concept of TEQs was created, and the toxic equivalency factor TEF for PCB people has been calculated for scientific regulations (Giesy & Kannan, 1998). Current studies, however, may not accurately reflect PCB transport to a target organ since pharmacokinetics, metabolism, and excretion are not taken into consideration. Furthermore, there is inadequate evidence on the potential biological and toxicological consequences of PCB metabolites, as well as restricted implementation of TEF in PCB metabolite risk assessment (Herrero et al., 2022).

By interacting with several enzymes, oxidative stressors play a key role in the harmful action of PCBs. Furthermore, PCBs produce ROS along their metabolic process. PCBs disrupt antioxidant defense enzymes and signaling pathways, resulting in secondary oxidative stress. Because of the presence of highly reactive functional groups on PCB metabolites, there is growing evidence that they may have a greater hazardous potential than their corresponding parents (Perkins et al., 2016). According to Mariussen et al., PCB 153 and the combo of Aroclor 1254 and Aroclor 1242 raised ROS levels 1.5- and 1.8-fold higher than controls (Mariussen et al., 2002), respectively, and Dreiem et al. showed that hydroxylated PCB exposure increased ROS levels more than 10-fold higher than the control group (Dreiem et al., 2009).

2.2.8. Metabolism of PCBs

To properly comprehend PCB toxicity or examine the role of metabolism in toxicity, it is vital to understand the behavior of PCB metabolites in biological systems. PCB metabolites may play important roles in detrimental human health impacts, although their importance is frequently overestimated. In this situation, it is critical to treat the hazardous consequences of PCB metabolites. Although many of the physiological impacts of parent PCBs are receptor-mediated, such as the well-known properties of PCBs as inducers of xenobiotic metabolism, additional PCB toxication pathways involve the metabolism of PCBs or their metabolic offspring (Grimm et al., 2015a).

Many factors influence PCB metabolism, including chemical structure and characteristics, solubility, concentration, and temperature, among others (Grimm et al., 2015b; Safe, 1993). Low-chlorinated PCBs are more susceptible to metabolism because of the fewer chlorine atoms on biphenyls, particularly the chlorine atoms on meta- and para position. In contrast, because insoluble highly chlorinated PCBs are resistant to metabolism, they are more likely to accumulate in adipose tissue due to their lipophilic nature (Hutzinger et al., 1974; Safe et al., 1974). The inclusion of vicinal non-chlorine substituted sites, notably in the biphenyl core's meta- and para-positions, enhances the chance of CYP-mediated transformation.

2.2.8.1. Metabolic pathways leading to hydroxylates and quinone type metabolites

The initial step of metabolism starts with the oxidation of PCBs into OH-PCBs by hepatic p450 cytochrome enzymes. Three pathways are associated with monohydroxylated PCBs formation i.e. arene oxide-dependent, arene oxide-independent, and NH shift rearrangement reactions (Daly et al., 1972; Gardner et al., 1973). The CYP450 family mediates the arene oxide-independent way by direct insertion in meta-position. The arene oxide method requires CYP450 to stimulate the synthesis of an epoxide intermediate chemical, which is subsequently converted to a hydroxylated compound through a keto-enol tautomer reaction. The PCB arene oxide intermediate can spontaneously undergo a "NIH shift" rearrangement process, causing chlorine atom(s) to relocate to neighboring locations, complicating downstream metabolites even further. Another metabolic route that is not dependent on arene oxide exists. Hydroxylated PCB metabolites are generated by directly introducing a hydroxyl group into parent PCBs (Ariyoshi et al., 1995; Gardner et al., 1973; Hutzinger et al., 1972; Liu et al., 2020b).

PCB metabolism and the region selectivity of the initial arene oxide formation are affected by several factors, including, but not limited to, the degree of ring chlorination, the chlorine ring substitution pattern, the subfamily and levels of CYP450 isozymes, and other drug-metabolizing enzymes in the target organ. The rate of PCB metabolism is largely dependent on the degree and locations of chlorination; the more metabolized PCBs have neighboring unsubstituted carbon atoms in the para- or meta-positions. PCB metabolism rates vary widely between species. When compared to birds and fish, mammals metabolize these chemicals the fastest, however, rates of metabolism vary widely among mammalian species (Matthews et al., 1984). Different CYP superfamily enzymes may metabolize structurally distinct PCB congeners. Non- ortho-substituted PCB congeners (also known as co-planar or dioxin-like) are mostly processed by the CYP1A enzymes, whereas numerous ortho-substituted PCBs are substrates for the CYP2B enzyme (Lu et al., 2013; Lu et al., 2011).

When dihydroxylated metabolites have hydroxyl groups that are ortho- or para- to each other, peroxidases may catalyze oxidation to ortho- or paraquinone, resulting in the formation of PCB semiquinone radicals via one-electron oxidation/reduction of hydroquinones and quinones (Wangpradit et al., 2009). Semiquinone's stability is strongly reliant on the substituents on the quinone ring. Because of the unpaired electrons,

semiquinone metabolites are significantly more reactive. They prefer to receive or lose one electron and produce the equivalent hydroquinone and quinone. Furthermore, one electron transfer converts two molecules of semiquinone into one molecule of quinone and one molecule of hydroquinone (Wangpradit et al., 2009).

2.2.8.2. Metabolic pathway leading to sulphur containing metabolites

PCBs are further converted into CB methyl sulfones (MeSO2), sulfate, and glucuronic acid conjugates through the conjugation pathway to accelerate the elimination of exogenous PCBs from the body. The metabolism using the mercapturic acid pathway first accomplishes the catalyzed reaction through the conjugation of arene oxide PCBs to GSH by GST. The glutamyl residue is then transferred by GGT, while the glycine residue is cleaved by cysteinyl glycinase. Mercapturic acid is formed during N-acetylation of cysteine. The C-S bond of cysteine is then cleaved by lyase, a pyridoxal phosphate-dependent enzyme, yielding a thiol derivative. Thiol derivatives methylated using S-adenosylmethionine as a methyl group donor generate a corresponding methyl sulfide, which is then sulfoxidized to aryl methyl sulphones in a two-step CYP-catalyzed process (Bakke & Francis, 1989).

The rate of generation of MeSO2-metabolites is mostly determined by the degree of chlorination, with lower chlorinated congeners (tri- or tetra-) metabolizing faster than higher chlorinated ones (penta- or hexa-). It is worth noting that a greater degree of PCB 3-methylsulfonyl compounds have a reasonably high affinity in liver and adipose tissues, whilst 4-methylsulfonyl derivatives have a pretty high affinity in lungs. This discovery is also consistent with previous studies demonstrating the preponderance of metasubstituted congeners in the liver and para-substituted congeners in the lungs (Larsson & Bergman, 2001; Norström et al., 2006). Hamsters generated small quantities of meta- and para-MeSO2 metabolites, but guinea pigs and rats produced substantially larger levels of MeSO2 metabolites. The results demonstrated that meta-substituted MeSO2- metabolites produced from PCBs with 2,3,6-chlorine substitution had a preferential distribution in guinea pigs, showing that the meta-position of the 2,3,6-trichlo-substituted phenyl ring in the molecule is the preferred location for methylsulfonylation. These results show that

the synthesis of methylsulfonyl PCB metabolites is species-dependent and also related to the chlorine substitution pattern (Liu et al., 2020b).

2.2.8.3. Metabolic pathway leading to glucuronic acid metabolites

Multiple oxidation processes have also been seen in OH-PCBs, resulting in more than one hydroxyl substituent on the biphenyl structure. OH-PCBs are also substrates for conjugation processes mediated by SULTs or UDP- UGTs to give their respective sulfate or glucuronic acid conjugates, which exist as a mediator in human, rat, mouse, fish, or monkey microsomes. This is a phase II metabolism that produces hydrophilic molecules that aid in the excretion of PCBs. Furthermore, UGT-catalyzed glucuronidation is tissue and PCB structure-dependent. That is, the metabolic efficiency of hydroxylated PCBs is determined by the quantity of chlorine atoms on biphenyls. This reaction preferentially occurs in low molecular weight PCB congeners (with fewer chlorine substituents) because of the comparatively large size of glucuronic acid (Liu et al., 2020a).

According to recent research, hydroxylated and sulfated PCB metabolites are inhibitors or substrates of SULTs and UGT. Because these enzymes are involved in the metabolism of alcohols, steroid hormones, bile acids, and other substances, hydroxylated PCBs have been linked to immunological, cardiovascular, metabolic, and neurological dysfunctions. Interestingly, unlike hydroxylated PCBs, PCB quinones have a distinct role in SULT catalysis. Lower PCB quinone concentrations improved SULT catalytic activity, but larger amounts hindered catalysis. These alterations in SULT catalytic efficiency may also have an impact on steroid hormone and metabolism of bile acid (Ekuase et al., 2011; Parker et al., 2018; Qin et al., 2013)

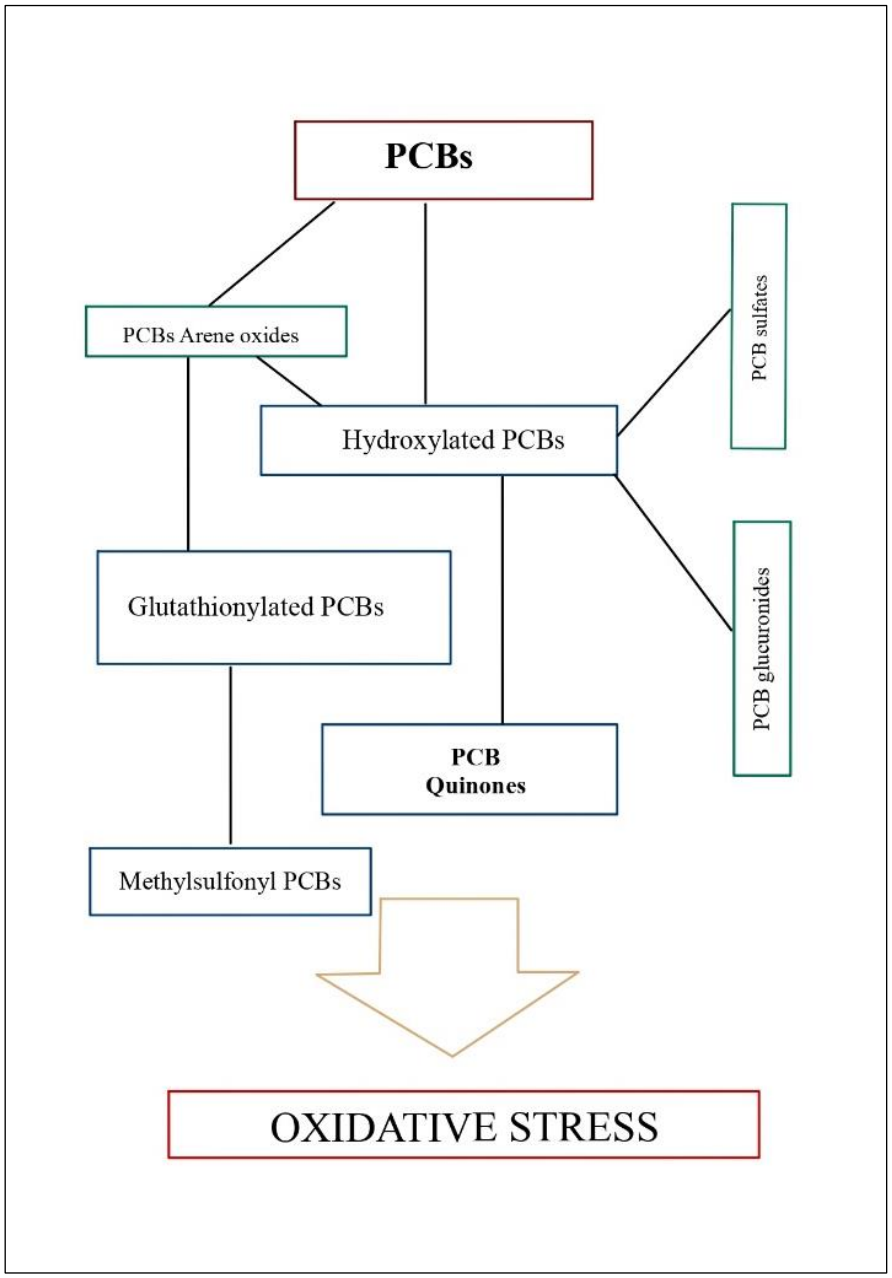

Figure 2.3. Metabolism of PCBs; The parent PCBs are metabolized by the aid of cytochrome P-450 monooxygenases (CYPs) to hydroxy PCBs. The hydroxylated PCBs further go through sulphation and glucuronidation to form PCB sulfates and glucuronides respectively via SULTs and UGTs. While during other steps hydroxylated PCBs are metabolized to form quinones and methyl sulphonyl PCBs, which are the main candidates for causing oxidative stress. SULTS; Sulfotransferases, UGTs; UDP- glucuronosyltransferases.

2.2.9. PCBs and oxidative stress

ROS are formed as a consequence of cellular metabolic pathways and serve as an important second messenger in many intracellular signaling pathways. On the other hand, excessive intracellular ROS production causes oxidative stress in a cell. PCBs and their metabolites have been linked to a variety of toxicological effects, including apoptosis, autophagy, pyroptosis, DNA damage, lipid peroxidation, metabolic failure, and redox-enzyme imbalance. PCB exposure is well-accepted to be linked to ROS formation, GSH depletion, lipid peroxidation augmentation, and antioxidant enzyme activity suppression (Liu et al., 2020a).

PCBs cause oxidative stress by interacting with aryl hydrocarbon receptors (AhR) and activating the cytochrome P450 1A subfamily; particularly coplanar PCBs have a high affinity for this mechanism. In comparison, PCB 153, a non-AhR ligand, had negligible or low oxidative stress potential. Several studies have also suggested that ROS generation may be caused by mitochondrial malfunction. In addition, PCB-driven ROS generation is NADPH oxidase-dependent.

Because of PCB metabolism, ROS are continually created. Microsomal enzymes may convert PCBs to mono and dihydroxylated intermediates, which can then be oxidized to quinones. A redox cycle may be created between PCB hydroquinone and semiquinone, and ROS can be produced during the redox reactions of PCB metabolites in vitro. ROS, lipid peroxidation, protein carbonyl level, and 8-OHdG seemed to be consistently high, suggesting oxidative stress. After being exposed to PCBs, antioxidant enzymes were promptly activated to remove excess ROS (Liu et al., 2020a).

PCBs have been categorized as class I human carcinogens by the IARC; nevertheless, it is unclear whether their metabolites contribute to carcinogenicity. Few research has looked at how PCB metabolites (mostly hydroxylated and quinone PCBs) act as carcinogen initiators, promoters, or co-carcinogens (Dhakal et al., 2018). Various PCBs and metabolites have a genotoxic influence on the order of parent PCBs, which is consistent with their ability to produce ROS i.e., parent PCB < mono hydroxylated PCB < dihydroxylated PCB < PCB quinone, suggesting that quinones may be the ultimate carcinogen (Selvakumar et al., 2013).

2.2.9.1. Liver function abnormalities

Workplace exposure can result in extremely high body loads of these chemicals, and this human demographic is the most vulnerable. Various hepatic effects have been documented, including elevated blood levels of liver enzymes, particularly glutamyl transferase (GGT) and lipids, as well as enhanced hepatic drug-metabolizing enzymes. However, the specific PCB combination utilized, the quantity of exposure, the degree of contamination with PCDFs, and concomitant exposure to other chemicals differed between investigations. Aside from these obscuring variables, the findings imply that excessive PCB exposure alters liver function. It is unclear if this change is just due to the stimulation of liver metabolizing enzymes or if it has therapeutic importance. Few scientists have published data on mortality from liver cirrhosis due to the very modest size of the occupational groups exposed to PCBs (Brown & Jones, 1981; Chase et al., 1982; Emmett, 1985; Lawton et al., 1985).

2.2.9.2. Carcinogenesis

Several studies have documented or examined the occurrence of cancer in PCB-exposed employees. The results showed that no overall increases in cancer-related mortality could be linked to occupational exposure to PCBs in the cohorts with the highest number of employees. However, there are elevated occurrences of certain malignancies in some of these studies, including melanoma and the grouping of liver, gall bladder, and biliary system cancers, gastrointestinal tract cancer in men, and hematologic neoplasms. Some of the increases in cancer occurrences at specific sites were not statistically significant, and the carcinogenic effects reported in these employees varied among studies. These findings imply that PCBs do not produce a consistent rise in one or more malignancies in highly exposed workers, and so their carcinogenicity in humans has not been demonstrated. Another conclusion was that a high proportion of kidney malignancies were reported (Shalat et al., 1989). A higher-than-expected incidence of deaths from pancreatic cancer was also seen in the biggest of the PCB-exposed cohorts (Yassi et al., 1994).

2.2.9.3. Reproductive effects

The results of animal research and the experience in Taiwan and Japan with combined PCB/PCDF exposure motivated studies of pregnancy outcomes about PCB exposure. Women who were occupationally exposed to PCBs had children that were 60 g lighter than children from the same factory who were less exposed. This difference was explained in part by the shorter gestation (1/3 of a day) among those exposed. The relationship between birth weight, gestational age, and level of exposure has been inconsistent in populations less exposed to PCBs, with some studies showing lighter babies among those with greater exposure, others showing no effect, and still, others showing increased exposure was associated with slightly heavier babies (Longnecker et al., 1997).

2.2.9.4. Immunologic effects

The cutaneous delayed hypersensitivity reaction to mumps and trichophyton was studied in employees with high levels of PCB exposure and found to be no different than in a control group. When PCB exposure was continued in another set of employees, PCB levels were connected to greater lymphocyte counts; moreover, the makeup of the white cell population was related to exposure, although in an inconsistent manner (Emmett et al., 1988; Lawton et al., 1985).

2.2.9.5. Dermatologic effects

In addition to chloracne, occupational exposure to PCBs appears to produce a variety of skin problems. 10% of capacitor manufacturing employees, for example, developed hyperpigmentation (Fischbein et al., 1982).

2.2.9.6. Other effects

Following PCB exposure, certain populations have reported neurobehavioral dysfunction, high blood pressure, dark brown nail pigmentation, peculiar hair follicles, skin thickening, different ophthalmic issues, and numbness in some extremities (Kilburn et al., 1989; Kimbrough & Jensen, 2012; Kreiss et al., 1981).

2.3. Aroclor 1254

Aroclor 1254 is a polychlorinated biphenyl (PCB) mixture that has found widespread industrial application due to its outstanding electrical insulating qualities, chemical stability, and heat resistance. Aroclor 1254 is made up of different PCB congeners, with the "1254" prefix alluding to its chlorine level (54%). Aroclor 1254's particular congener composition may vary based on the production method and intended application (Haque et al., 1974).

Aroclor 1254 is a viscous liquid that ranges in hue from pale yellow to amber. Physical qualities like as density, boiling temperature, and solubility are determined by the congener makeup. The melting point is normally between -20°C and 5°C, while the boiling point is between 360-380°C. Aroclor 1254 is water insoluble but soluble in organic solvents such as benzene and toluene. Commercial PCB preparations, such as Aroclor 1254, are classified as mixed-type inducers because they comprise dioxin-like, mono-ortho, and di-ortho congeners and, as a result, may stimulate both CYP1A and CYP2B enzymes in laboratory animals (Ngui et al., 1999).

2.3.1. Aroclor 1254 harmful effects on liver

The harmful result of oxyradical and oxidant production and reactivity with biological components is oxidative stress. ROS (oxy radicals) are continually produced in the nervous system during normal metabolism and neuronal activity. Oxidative stress occurs when the equilibrium between the ROS and antioxidant systems is disrupted. The

free radical O2 is produced by a variety of enzymatic and non-enzymatic processes and is frequently the first step in the oxidative stress cascade.

Because the liver is the primary location for the detoxification of hazardous metabolites, it is thought to be a possible target for PCB-induced harm. In goat, rabbit, and duck livers, PCBs produced degradative alterations such as cytoplasmic loss and peripheralization of cytoplasm and organelles, according to Williams (Williams et al., 1993). PCBs have been found in several studies to induce lipid peroxidation in rat liver. Furthermore, PCBs have various harmful effects on the liver, and the mechanisms of toxicity are unknown.

Aroclor 1254 favors hepatotoxicity by induction of apoptosis via. upregulation of aryl hydrocarbon receptor and modulation of p53, and apoptotic protein (Bax, caspase-9, and caspase-3) and upregulating the antiapoptotic protein (Bcl-2) expression patterns (Pingili et al., 2020; Sekaran et al., 2012). Under some pathological situations, such as PCB exposure, mitochondria are a major intracellular generator of ROS as well as a vulnerable target for oxidative damage.

Cardiolipin, a tetra acyl phospholipid, is abundant in mitochondrial membranes. Its structural integrity is critical for successful oxidative phosphorylation. The rise in ROS production might be linked to mitochondrial malfunction. Furthermore, there is a substantial body of data supporting the hypothesis that mitochondrial dysfunction is a critical event in ROS-signaling via apoptotic pathways (Kirkland et al., 2003; Roué et al., 2003; Shih et al., 2004).

The activity of mitochondrial EROD and PROD are often utilized as enzymatic markers for hepatic CYP1A and CYP2B enzymes. According to Hamdy A.A. Aly, Aroclor 1254 (60 M) considerably boosted EROD (fivefold) and PROD (fourfold) activity when compared to matching controls. The activity of these two enzymes increased in a dose-dependent way. These findings stipulate that the Ahr (CYP1A) enzyme is required for Aroclor-induced mitochondrial superoxide generation in hepatocytes. Furthermore, CYP2B in hepatocyte mitochondria may be a target for Aroclor 1254 toxicity (Aly & Domènech, 2009).

Apart from these markers, mitochondrial aconitase activity has been evaluated as an enzyme that is inactivated by superoxide. Aconitase inactivation has been suggested as a reliable marker for mitochondrial ROS production (Hausladen & Fridovich, 1994).

Apart from this inhibition of complexes I and III of mitochondrial respiratory chain, increased lipid peroxidation, elevated LDH levels, decreased the enzymatic antioxidant GR and GPx activities and the non-enzymatic antioxidant GSH depicts that aroclor 1254 increased ROS generation in hepatocyte mitochondria in a dose-related manner (Aly & Domènech, 2009; Dröge, 2002; Hsiao et al., 2001; Kaufmann et al., 2005; Latipää et al., 1986). Cardiolipin levels were lower in Aroclor 1254-treated hepatocytes, which could account for the change in mitochondrial membrane lipid order, as cardiolipin is a mitochondrial anionic phospholipid known to confer stability and fluidity to the mitochondrial membrane. As a result, it is possible that ROS-induced oxidative damage to mitochondrial cardiolipin is to blame (Hauff & Hatch, 2006).

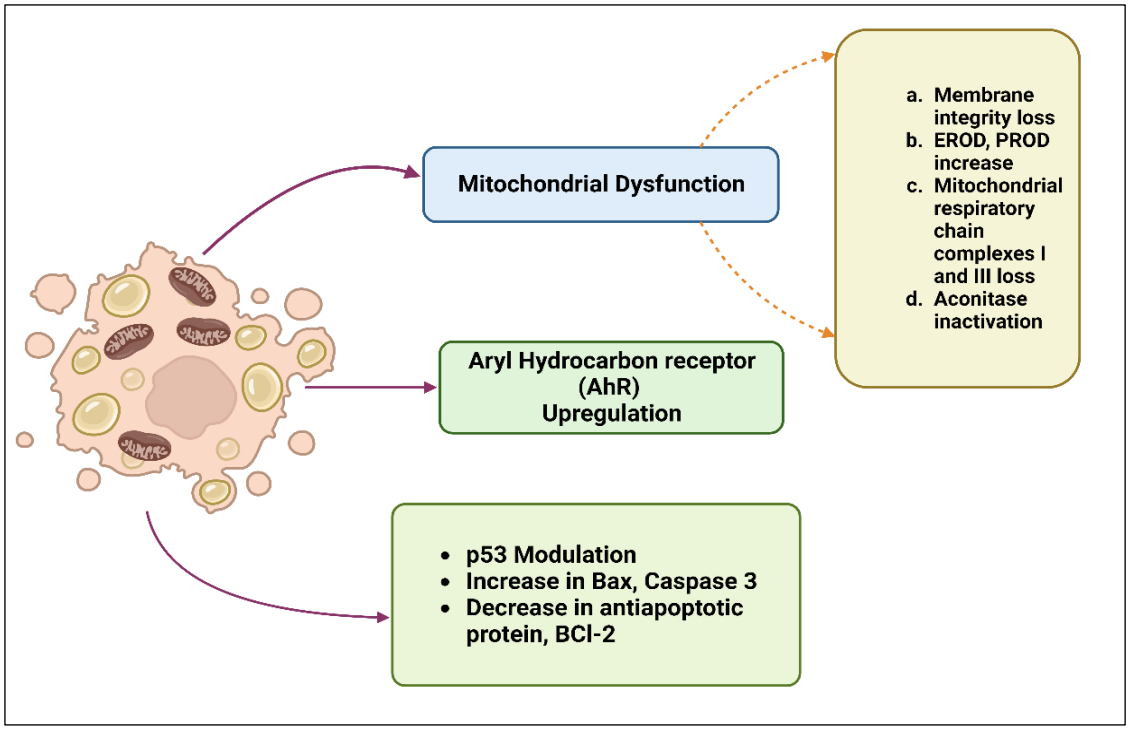

Figure 2.4. Possible cellular mechanisms through which A1254 can induce hepatotoxicity. 1. Mitochondrial dysfunction; loss of membrane integrity, increase in EROD and PROD, mitochondrial respiratory chain breakdown by inhibition of complex I and III, Aconitase inactivation. 2. Upregulation of AhR to induce tumor generation through different pathways. 3. Genetic disturbance; p53 modulation, induction of apoptotic proteins like caspase 3, Bax, and inhibition of antiapoptotic proteins like BCl-2. A1254; Aroclor 1254, EROD; ethoxyresorufin O-deethylase, PROD; pentoxyresorufin O-deethylase.

2.4. Terpenes

Human health benefits from exposure to the natural environment. Much research has emphasized the impacts of forest exposure on environmental exposures. Terpenes, the biggest class of naturally occurring organic chemicals with over 40,000 structures identified so far, are key components of the forest atmosphere (Gershenzon & Dudareva, 2007). Terpenes are the most abundant and diversified category of naturally occurring chemicals occurring mostly in plants. Larger terpene classes, such as sterols and squalene, can be found in animals. They oversee plant scent, flavor, and pigmentation. They can also be found in abundance in fruits, vegetables, and flowers. During and soon after blooming, their concentration is often high in plant reproductive structures and leaves. Terpenes are also a significant component of plant resins. They serve as info chemicals, attractants, or repellents in plants, and are responsible for the characteristic aroma of many plants. Terpenes, on the other hand, may be poisonous in large amounts and are thus an essential weapon against herbivores, pests, and diseases (Crowell & treatment, 1997; Dudareva et al., 2005; Theis & Lerdau, 2003).

2.4.1. Chemical properties

Terpenes are produced biochemically from isoprene units having the molecular formula C5H8. All terpenes have the same fundamental formula (C5H8) n, where n is the number of connected isoprene units (Gao & Singh, 1998). Isoprene units can be joined "head to tail" to create linear chains or grouped in rings. Terpenes can be hydrocarbons or molecules containing oxygen, such as hydroxyl, carbonyl, ketone, or aldehyde groups. Terpenoids are substances that come from the chemical alteration of terpenes (Paduch et al., 2007).

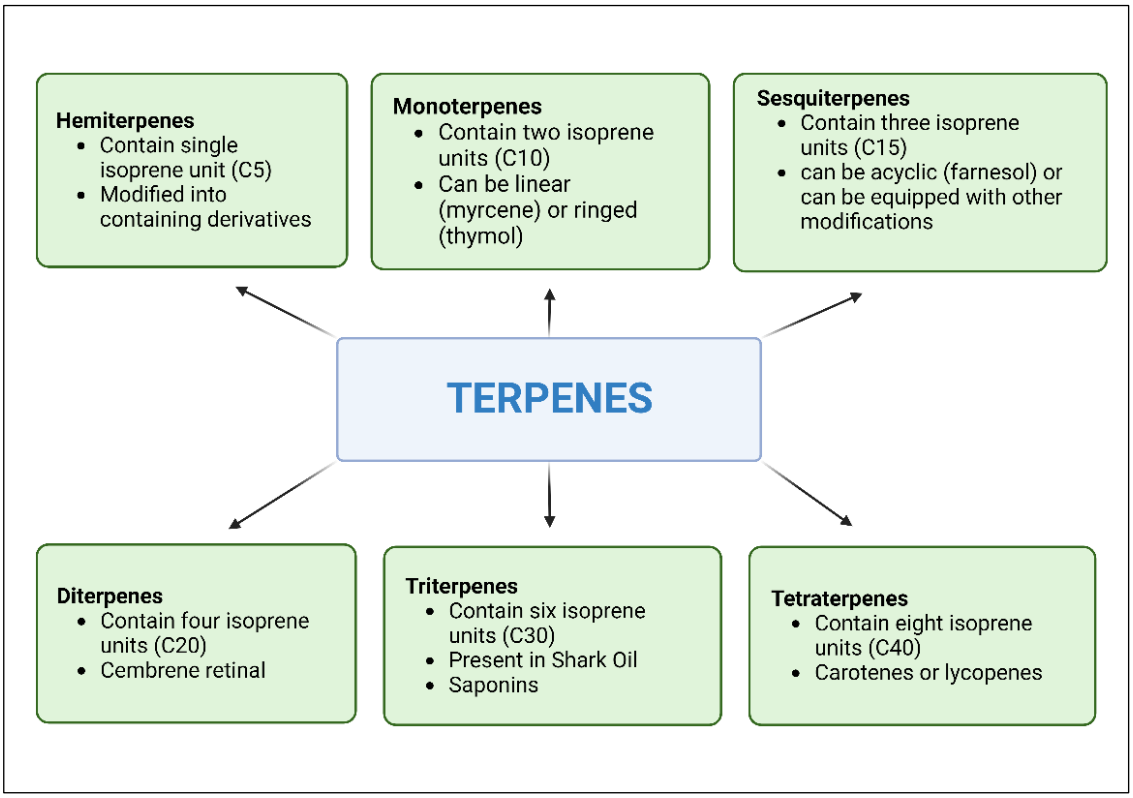

Figure 2.5. Classification of Terpenes. Terpenes are classified based on isoprene units. Hemiterpenes with single Isoprene unit (C5). Monoterpenes with two isoprene units (C10). Sesquiterpenes with three isoprene units (C15). Diterpenes with four isoprene units (C20). Triterpenes with six isoprene units (C30). Tetraterpenes with six isoprene units (C40).

2.4.2. Metabolic pathway

IPP and DMAPP, the universal isoprenoid precursors, are produced by two separate routes that developed in taxonomically diverse animals, named as mevalonic pathway (MVA) and methylerythritol phosphate pathway (MEP) (Pulido et al., 2012).

The MVA pathway, originating from archaea and present in eukaryotic cytosols, utilizes acetyl-CoA as a cellular endogenous substrate input. (Lohr et al., 2012). The MVA route is utilized by Archaea, bacteria, yeast, fungus, and mammalian cells, with genes organized into operons and thought to be transcribed (Hunter, 2007). It is in charge of synthesizing sterols and certain sesquiterpenes and may also be involved in synthesizing transhinones (Zhang et al., 2012).

Most bacteria, including photosynthetic bacteria, and chloroplasts use the MEP pathway. The isomeric IPP and DMAPP molecules are the end products of both pathways. For the MEP pathway, the cellular endogenous substrate inputs are pyruvate

and glyceraldehyde 3-phosphate. MEP is found in a variety of bacteria, including fermentative and aerobic bacteria, photosynthetic bacteria, cyanobacteria, micro- and macroalgal chloroplasts, and all plant chloroplasts. MEP, found in plants and algae's plastidic compartments, stimulates the production of primary isoprenoids like chlorophyll phytol tail, all carotenoids, and the prenyl tail of quinone molecules (Tetali, 2019). The development of antimicrobial drugs to target illnesses such as malaria and sexually transmitted infections is a critical application of this route (Hunter, 2007). Because this pathway does not exist in humans, it is a valuable resource for creating antibacterial and antiparasitic drugs (Seemann et al., 2009).

2.4.3. Therapeutic uses of terpenes

Some of the therapeutic actions of terpenes are listed below:

2.4.3.1. Anticancer uses

The medical benefits of terpenes are not limited. They are useful in a wide range of medical therapies. Terpenes are also extensively recognized for their anticancer effect. Research published in early 1997 indicated that a mixture of monoterpenes, diterpenes, and sesquiterpenes can successfully cure malignancies of the colon, brain, prostate gland, and bones. Epidemiological research suggests that edible monoterpenes may be useful in the prevention and treatment of cancer. D-limonene and perillyl alcohol, two dietary monoterpenes, have been found to have chemopreventive and therapeutic activities against a variety of human malignancies. Limonene is a physiologically active food component found in a variety of citrus fruits and peels (Tetali, 2019). The mechanism of limonene action is currently being investigated. According to one study, limonene works by inducing TGFB-1 and mannose-6-phosphate/insulin-like growth factor II receptors (Jirtle et al., 1993). One theory is that limonene kills cancer cells by inducing apoptosis (Rabi & Bishayee, 2009). In 2013, a study found that limonene inhibits breast tumor cyclin D1 expression, leading to cell cycle arrest and reduced cancer cell growth in early-stage breast cancer patients. (Miller et al., 2013). According to one study, pinecones

limonene can efficiently kill lung cancer cells in vitro via an apoptotic mechanism mediated by the caspase-3 pathway (T. K. Lee et al., 2017). These findings raise the possibility of limonene being used innovatively to combat and prevent cancer.

Metabolite of limonene, Perillyl alcohol is a naturally occurring monocyclic monoterpene with anti-tumor action in several pre-clinical tumor types (Crowell & treatment, 1997). Perillyl alcohol, like D-limonene, inhibits post-translational isoprenylation of tiny GTP-binding proteins, which is the foundation of its anticancer actions. Another mechanism of perillyl alcohol's chemotherapeutic actions is the stimulation of TGF-signaling. TGF-signaling activation by perillyl alcohol is related to increased production of pro-apoptotic proteins (Bax, Bak, and Bad) while having no effect on p53 or Bcl-2 expression (Ahn et al., 2003).

In addition to limonene, the terpene thymoquinone has been widely studied for its chemoprotective and chemotherapeutic properties. Thymoquinone is found to be an active component in the volatile oils of the annual herbaceous plant Nigella sativa (black cumin) (T. K. Lee et al., 2017). Thymoquinone affects the signaling pathways *p53, PPAR, MAPK, NF- κβ, PI3K/AkT,* and *STAT3* to exert its anticancer activities (T. K. Lee et al., 2017). Thymoquinone has been proven to be anti-cancer in studies on various types of cancer, including breast, skin, non-small cell lung, bile duct, and brain cancer. Apoptosis and cell cycle arrest are the fundamental processes underpinning cancer inhibition of thymoquinone (Khader & Eckl, 2014; Sobral et al., 2014).

Terpenes, natural molecules with minimal side effects, are gaining interest in cancer treatment due to their potential as a new class of medicines in modern oncology, potentially licensed as new drugs.

2.4.3.2. Antivirals uses

Novel viral infections prompt a search for antiviral medicines, with monoterpenes being the most effective, as they consist of two isoprene units.

The oil extracts were investigated for their antiviral activity against three main human viruses: HSV1, dengue virus type 2, and Junin virus. The virucidal oils predominantly comprised monoterpenes like carvone, carveol limonene, alpha and beta-

pinene, caryophyllene, camphor, beta-ocimene, and germacrene, a sesquiterpene (Duschatzky et al., 2005). The mechanism behind the virucidal action has been proposed as the direct inactivation of unbound virus particles. However, the study revealed that a combination of monoterpenes is more efficient and less hazardous to host cells than isolated single monoterpenes (Astani et al., 2010). This was supported further by research that examined the activity against a human flavivirus called West Nile virus. Both in vivo and in vitro outcomes were good. The fundamental mechanism was believed to be actuated G0 or G1 cell cycle halt (Zamora et al., 2016). The study demonstrated that a combination of monoterpenes has a more potent antiviral effect than a single monoterpene, as seen with the reduction of the RSV when exposed to triketone-terpene adducts obtained from Myrtaceae secondary metabolites such as sesquiterpenes myrtucomvalones A, B, and C. (M. Chen et al., 2017).

Researchers are also trying to develop terpene hybrids derived from fungal sources, which are speculated to have antiviral and UV-protective capabilities (Yuan et al., 2017). To recapitulate, terpenoids are an engaging class of natural compounds that have both particular and widespread antiviral activity and may be employed to amplify the therapeutic effectiveness of current antiviral therapy.

2.4.3.3. Antimicrobial and antifungal uses

Terpenes with antimicrobial characteristics, or the capacity to kill or inhibit the development of microorganisms, are widely employed in traditional and modern medicine (Himejima et al., 1992). Bacteria, as well as fungi, were tested (Trombetta et al., 2005). It has been demonstrated that microbes have a distinct sensitivity to plant-derived chemicals. Terpenes are more susceptible to Gram-positive bacteria than Gram-negative bacteria (El-Sawi et al., 2009). This is mostly driven by changes in the permeability, content, and charge of the microbes' exterior structures. Terpenes' antibacterial activity mechanism is directly related to their lipophilic nature.

Monoterpenes predominantly modify membrane structures, increasing membrane fluidity and permeability, altering membrane protein topology, and causing disruptions in the respiration chain (Trombetta et al., 2005). Furthermore, studies against the Gram-positive bacterium Staphylococcus aureus yielded good results. The antibacterial activity

of terpenes against S. aureus were ranked as follows: farnesol > (+)-nerolidol > plaunotol > monoterpenes such as (-)-citronellol, geraniol, nerol, and linalool. Thymol and (+)-menthol (monoterpenes) showed substantial toxicity when tested against S. aureus, while (+)-menthol was highly hazardous against Escherichia coli (Hada et al., 2003).

Plants that generate antimicrobial terpenes include *Pinus ponderosa* (Pinaceae), spices (sage, rosemary, caraway, cumin, clove, and thyme), *Cretan propolis, Helichrysum italicum, Rosmarinus officinalis*, and others. Utilizing these antimicrobial terpenes can help combat food-borne pathogens such as *E. coli, Staphylococcus aureus*, and *Bacillus cereus* (Himejima et al., 1992). Diterpenes, 14,15-dinor-13-oxo-8(17)-labden-19-oic acid, and a combination of labda8(17),13E-dien-19-carboxy-15-yl oleate, palmitate, and triterpene may all be separated from Cretan propolis (Popova et al., 2009).

The structures of the various substances have been determined using spectroscopic research and chemical evidence. These terpene-derived compounds were investigated for antibacterial activity against bacteria such as gram-positive and gram-negative bacteria (Popova et al., 2009). All of it was evaluated for human pathogenic fungi with broad-spectrum antibacterial action (Popova et al., 2009). A wide range of sesquiterpenes and lactones were also shown to be active against Mycobacteria. Furthermore, antimycobacterial action was demonstrated by sesquiterpene lactones such as costunolide, parthenolide, and others. Among the triterpenes that tested positive for *M. intracellulare* and Gram-positive and Gram-negative bacteria were oleanolic acid and ursolic acid (Copp, 2003; Liu, 2005; Paduch et al., 2007).

Terpenes have antifungal properties as well. Experiments were carried out on two types of fungus: saccharomycetes and mildew fungi, both of which are potentially dangerous to humans. Both optical isomers of carvone were discovered to be efficacious against a wide range of human pathogenic fungi. Carvone and perillaldehyde impeded the transformation of Candida albicans from coccal to filamentous form, thereby enhancing the fungus's pathogenicity (De Carvalho & Da Fonseca, 2006). *Candida albicans, Candida krusei,* and *Candida tropicalis'* growth was hindered by a monoterpene composition containing terpinen-4-ol, α-pinene, β-pinene, 1,8-cineole, linalool, and α-terpineol (El-Sawi et al., 2009; Hammer et al., 2003).

In general, antifungal treatment based on terpenes and their derivatives is extremely promising, but it may be difficult to implement due to the large dosages of terpenes or terpenoids required. As a result, such therapy may have substantial adverse

effects. Nonetheless, these compounds may function as supplemental agents, potentially improving current antifungal therapy.

2.4.3.4. Anti-inflammatory uses

1,8-cineole (eucalyptol), a monoterpene derived from eucalyptus oil, inhibited arachidonic acid metabolism and pro-inflammatory cytokine production in activated human monocytes (Peana et al., 2003). Although 1,8-cineole was easily carried into tissues, it was released slowly into the plasma. It also had a long terminal half-life, thus it persisted in high quantities in the tissues even after the dose had finished. As a result, this monoterpene has been proven to be particularly beneficial in treating chronic diseases such as bronchitis, sinusitis, and steroid-dependent asthma, as well as a preventative agent against recurring respiratory infections (Peana et al., 2003).

Cucurbitacin R has been found to decrease *iNOS, COX-2, TNF-α,* and PGE2 levels in paw homogenates of rats with adjuvant-induced arthritis (Escandell et al., 2007). Furthermore, The naturally occurring diterpene pepluanone from Euphorbia peplus has been reported to lower *NO*, *PGE2*, and *TNF-α* production by inhibiting *COX-2* and *iNOS* activity and suppressing *NF-κβ* (Corea et al., 2005). Natural *NF-κβ* inhibitors have also been discovered in diterpenes (excisanin, kamebakaurin), triterpenes (avicin, oleandrin), sesquiterpenes and sesquiterpene lactones (costunolide, parthenolide), pentacyclic triterpenes (celastrol, celaphanol A), and pentacyclic triterpenes (aglycones of saponins) (Nam, 2006; Ralstin et al., 2006; Safayhi & Sailer, 1997). AKBA, isolated from Boswellia serrata, displayed significant anti-inflammatory action among the pentacyclic terpenoids. It selectively inhibits 5-lipoxygenase via a pentacyclic structure-binding site distinct from the substrate-binding site. As a result, AKBA is regarded as an anti-inflammatory treatment.

Other terpenes with anti-inflammatory effects in vitro and in vivo, mostly through decreasing PLA2 activity, have also been identified. PLA2 is significantly linked to the regulation and control of inflammatory processes involving arachidonic acid metabolism and phospholipid turnover (Máñez et al., 1997). Phospholipase Cβ, on the other hand, was inhibited by numerous sesquiterpenes, the most potent of which was petasin (Thomet et al., 2001). Betulin, betulinic acid, and ursolic acid extracted from Diospyros

leucomelas reduced inflammation in the carrageenan and serotonin systems, respectively, evaluation for paw edema (del Carmen Recio et al., 1995). Furthermore, oleanolic and ursolic acids have a hepatoprotective impact at low dosages, which may be owing to their anti-oxidant and anti-inflammatory properties. These triterpenoids are also excellent inducers of metallothionein, a tiny cysteine-rich protein that functions similarly to glutathione in the body's defense against a variety of natural and chemical stressors that can cause liver damage (Liu, 2005).

2.5. 1,8 Cineole

Natural plant resources are one of the most significant sources of potential medication developments. A chemical isolated from a specific section of the plant may have considerable pharmacological effects, making it a good candidate for research in the clinic. 1,8-cineole (cineole; eucalyptol), a monoterpenoid found in the essential oils of eucalyptus, rosemary, and psidium plant species, has long been used to alleviate symptoms of different diseases like respiratory infections by inhibiting the growth of several microorganisms (Santos et al., 2001).

As a saturated monoterpene, 1,8-cineole was mostly derived from plant essential oils such as Eucalyptus (Ogunwande et al., 2003), Salvia lavandulifolia (Porres-Martínez et al., 2015), and Melaleuca quinquenervia (Porres-Martínez et al., 2015). The greatest concentration of 1,8-cineole has been found in Eucalyptus essential oil. 1,8-cineole is commonly used in flavored, cosmetic, or fragrance items such as bath additives, mouthwashes, and insect repellents due to its pleasant aroma and taste. In terms of 1,8 cineole (eucalyptol) characteristics, it has a fresh camphor-like odor and a spicy, cooling taste. It is water-insoluble yet miscible with organic solvents. Eucalyptol accounts for around 70-90% of eucalyptus oil. The chemical structure of 1,8 cineole is elaborated in figure 2.6.

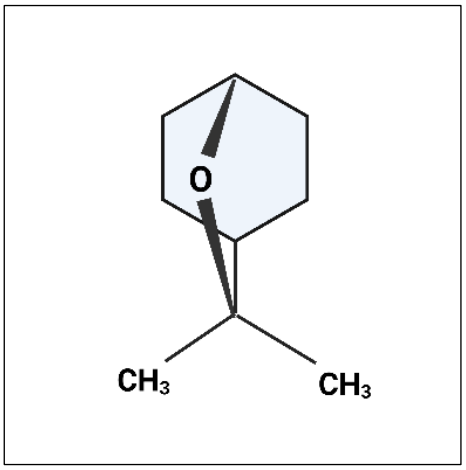

Figure 2.6. Chemical structure of 1,8 Cineole

2.5.1. Pharmacological action

The main pharmacological characteristics of 1,8 cineole are anti-inflammation and anti-oxidation.1,8-cineole reduces the synthesis of inflammatory cytokines and ROS by modulating the *NF-κβ* and *Nrf2* pathways, respectively (Z. Jiang et al., 2019). Clinical trials for 1,8-cineole have begun. Although 1,8-cineole has a promising preclinical profile in pharmacology studies, its low stability limits its clinical applicability. The creation of appropriate formulations is critical for further in-vivo use. 1,8 cineole has an intriguing pharmacological profile, with the capacity to treat cardiovascular disease, digestive illness, Alzheimer's disease (AD), and respiratory diseases (Cai et al., 2021).

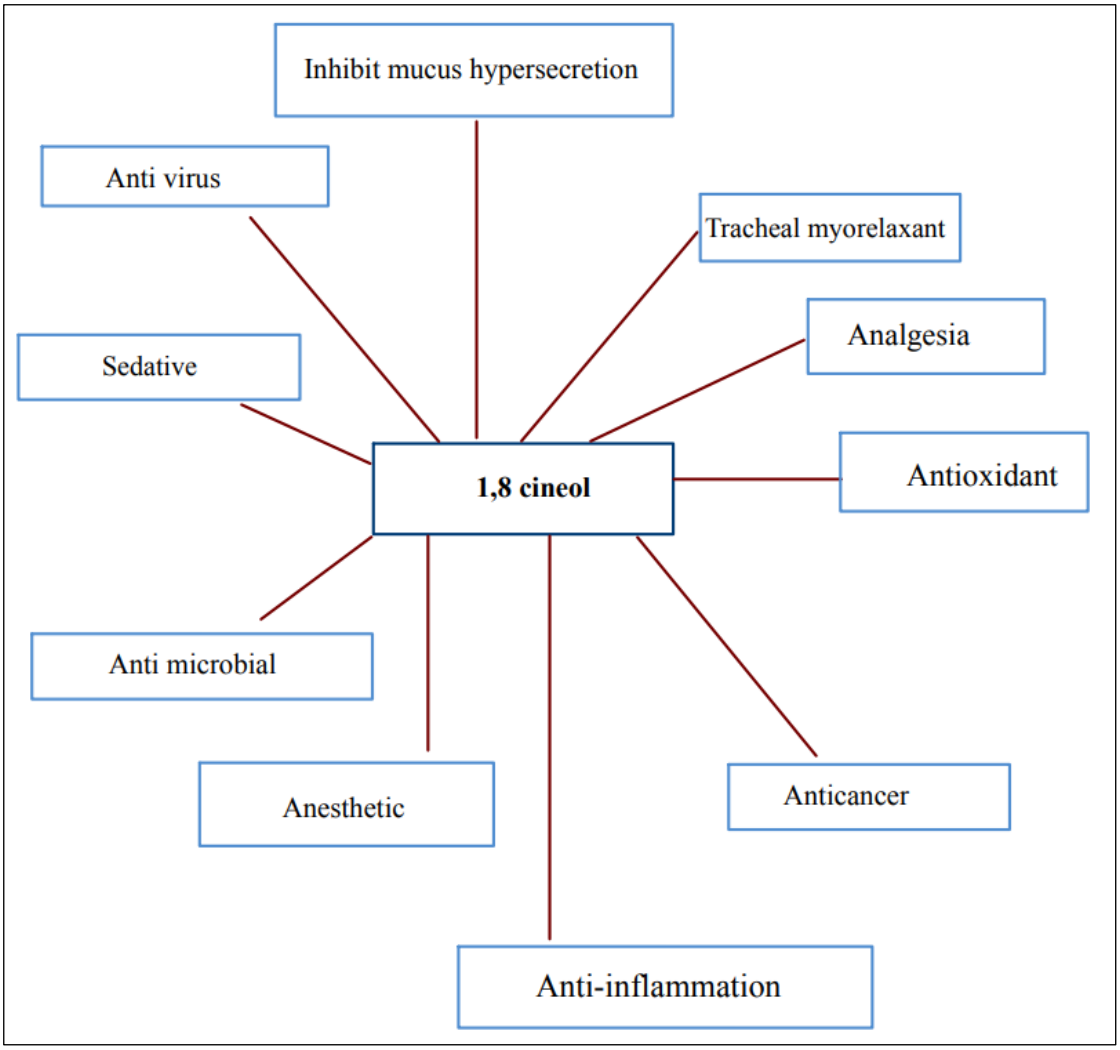

Figure 2.7. The possible therapeutic effects of 1,8 Cineole

2.5.2. Biological actions and mechanisms of 1,8 Cineole

For decades, essential oils containing 1,8-cineole have been utilized as traditional remedies. Furthermore, various studies have shown that 1,8-cineole, as an active molecule, is useful in the treatment of respiratory ailments, malignancies, digestive problems, dysphoria, Alzheimer's disease, cardiovascular illnesses, and bacilli.

2.5.2.1. Respiratory diseases

Many respiratory disorders, including influenza, bronchitis, rhinosinusitis, pneumonia, asthma, and COPD, have therapeutic benefits with 1,8-cineole, which has entered clinical studies in some situations (Cai et al., 2021). As an adjuvant for asthma and COPD, 1,8-cineole improved lung function, respiratory status, and quality of life in patients (Worth & Dethlefsen, 2012; Worth et al., 2009). Furthermore, 1,8-cineole demonstrated cross-protection against influenza virus when paired with vaccination (Y. Li et al., 2016) or oseltamivir (Lai et al., 2017) in mice, extending survival time and reducing weight loss, mortality, and lung damage. Other than that, 1,8-cineole alone significantly reduced airway and lung injuries (Bastos et al., 2011), protected against pathogenic viruses (Yang et al., 2010), and relaxed airway muscles (Nascimento et al., 2009). The anti-inflammation (Yu et al., 2018), muscle relaxation (Bastos et al., 2009), and mucus hypersecretion inhibition (Sudhoff et al., 2015) effects of 1,8-cineole in the treatment of respiratory diseases resulted in the downregulation of inflammation cytokines such as *IL-1β* and *TNF-α* (U. R. Juergens et al., 2004). Several studies have shown that the anti-inflammatory effect of 1,8-cineole may be linked to an important nuclear transcription factor *NF-κβ*. 1,8-cineole inhibited Iκβα degradation, thereby suppressing *NF-κβ* p65 translocation and decreasing the expression of pro-inflammatory *NF-κβ* target genes (Greiner et al., 2013). Other signaling pathways that can be triggered by 1,8-cineole to decrease inflammatory cytokine release include the p38 *MAPK*, protein kinase B (*Akt*), *TREM-1*, and *NLRP3* inflammasome (Lee et al., 2016; Yadav & Chandra, 2017). 1,8-cineole also inhibits the production and nuclear internalization of early growth response factor-1 (Egr-1) (ZHOU et al., 2007). The mechanism of 1,8-cineole's tracheal myorelaxant action may be linked to the blocking of L-type voltage-gated calcium channels (Pereira-Gonçalves et al., 2018). 1,8-cineole inhibited rhinosinusitis and bacteria-induced mucus hypersecretion, which is often associated with mucin expression (Sudhoff et al., 2015). Furthermore, 1,8-cineole was used to treat virus-induced respiratory illnesses by suppressing the N-protein and increasing the activity of the *IRF3* (Cai et al., 2021).

Most studies on the therapeutic mechanisms of 1,8-cineole have focused on its ability to reduce inflammation in respiratory diseases via multiple signaling pathways,

which has resulted in the improvement of pathophysiological features in chronic airway diseases such as pulmonary vascular changes and abnormal mucus production.

2.5.2.2. Digestive diseases

1,8 Cineole can also help with colonic damage, acute pancreatitis, non-alcoholic steatohepatitis, diarrhea, and gastric or liver damage (Lima et al., 2013; Santos et al., 2004). Notably, the antispasmodic and antisecretory properties of 1,8-cineole prevented castor oil-induced diarrhea (Jalilzadeh-Amin & Maham, 2015). However, three pathways may be implicated in 1,8-cineole's gastroprotective impact: the healing property to promote stomach cell regeneration, the cytoprotective effect to enhance gastric mucus, and the antioxidant activity to lower MPO activity (Jalilzadeh-Amin & Maham, 2015). Reduced levels of MPO and pro-inflammatory cytokine suggested that 1,8-cineole had antioxidant and anti-inflammatory effects in the treatment of trinitrobenzene sulfonic acid-induced colitis and cerulean-induced acute pancreatitis. So far, the methods of therapy for digestive illnesses by 1,8 cineole have mostly focused on antioxidant benefits, with further research focusing on anti-inflammatory activities related with *NF-κβ*. Although 1,8 cineole has been shown to protect digestive organs against inflammatory mediators and oxidative stress, it has not yet been licensed for clinical studies (Rocha Caldas et al., 2015).

2.5.2.3. Alzheimer’s disease

AD is an irreversible and progressive brain disease that primarily affects the elderly. The major characteristics of Alzheimer's disease (AD) are extracellular Aβ plaque formation and intracellular neurofibrillary tangles (Rubio-Perez & Morillas-Ruiz, 2012). Increasing data suggested that Aβ deposition caused the inflammation. It was confirmed that 1,8-cineole improved Ab25-35 in pheochromocytoma cells, and the anti-inflammatory mechanism may be connected to NF-κβ. Given that oxidative stress is a factor in the etiology of Alzheimer's disease, the antioxidant activity of 1,8-cineole may aid in therapy (Seo et al., 2018). The balance of oxidant/antioxidant effects was

maintained after pre-treatment with 1,8-cineole through Nrf2-activated ROS scavenging actions (Kaufmann et al., 2011). However, studies have shown that 1,8-cineole-rich plant extract was more effective than pure 1,8-cineole in the prevention of AD, which may be attributed to the multiple components in the natural extract achieving synergistic effects (Paul et al., 2020). Based on previous research, 1,8-cineole is a promising drug to prevent and treat AD through anti-inflammatory, antioxidant, and anti-AChE effects. However, the studies are limited to in vitro studies.

2.5.2.4. Cardiovascular diseases

The effects of 1,8-cineole on cardiovascular diseases, such as hypertension, ischemic stroke, and atherosclerosis, may pertain to blood pressure reduction, blood vessel relaxation, improved reverse cholesterol transport, resistance to cortical cell damage, amelioration of vascular endothelial injury, and depression of myocardial contractility (Cho, 2012; Linghu et al., 2019; Ryu et al., 2014). Antioxidant and anti-inflammation processes are mostly used in the treatment of cardiovascular disorders. In the cerebral ischemia damage model, 1,8-cineole was thought to be a ROS scavenger and an enhancer of SOD activity (Ryu et al., 2014). Furthermore, 1,8-cineole protected HDL from oxidative damage and increased HDL activity, consequently increasing reverse cholesterol transport (Cai et al., 2021; Cho, 2012). 1,8-Cineole inhibited *NF-κβ, p65* phosphorylation and prevented *NF-κβ* translocation from the cytoplasm into the nucleus, resulting in reduced inflammatory cytokine production and decreased *NO* overexpression, which improved endothelial cell damage (Linghu et al., 2016b).

Taken together, these findings support the idea that 1,8-cineole might be a viable drug in the treatment of cardiovascular disorders, particularly atherosclerosis. The anti-inflammatory and antioxidant properties of 1,8-cineole help to explain its considerable therapeutic impact on atherosclerosis.

2.5.2.5. Anticancer effects

Researchers have shown a strong interest in finding anti-cancer compounds in natural plants in recent years. According to published research, 1,8-cineole has been validated as a possible anticancer agent in vitro and in vivo against leukemia (Moteki et al., 2002), skin (Sampath et al., 2017), oral (Cha et al., 2010), colon (Murata et al., 2013), breast (Efferth et al., 2011), liver (Rodenak-Kladniew et al., 2020), and ovarian cancer (Wang et al., 2012). The anticancer activity is mostly due to the induction of cancer cell death via the tumor suppressor protein p53 signaling pathway. 1,8-Cineole was shown to successfully cause apoptosis and G2/M phase arrest in skin cancer cells by upregulating the expression of the p53 protein, while having no effect on normal keratinocytes (Sampath et al., 2017). In the treatment of human oral epidermoid carcinoma cells, 1,8-cineole triggered death via a caspase-dependent mechanism as well as a *MAPK*-mediated pathway (Murata et al., 2013). Furthermore, 1,8-cineole can cause apoptosis in human colorectal carcinoma by activating p38 *MAPK* as well as *Akt*, resulting in cleaved *caspase-3* and *PARP* (Murata et al., 2013).

It was recently shown that 1,8-cineole might influence the *AMPK*, Akt/mammalian target of rapamycin *mTOR*, and *MAPK* pathways to promote G0/G1 arrest and senescence of hepatocellular carcinoma cells (Kladniew et al., 2020). However, 1,8-cineole has only been shown to protect against UV-induced skin cancer. In vitro, the anticancer effects of 1,8-cineole have been demonstrated primarily via the *MAPK* and *PI3K*/*Akt*/*mTOR* signaling pathways (J. Lee, S. J. Ha, J. Park, Y. H. Kim, N. H. Lee, Y. E. Kim, Y. Kim, K.-M. Song, & S. K. Jung, 2017).

3. MATERIALS AND METHODS

3.1. Chemical Substances

The required chemical substances were 1,8 cineole, Aroclor 1254. 1,8 cineole, 99% with molecular weight 154.25 g was considered reliable for the experimental protocol in solution form as (1g/1ml; 100 gr of 1,8 cineol in total). Two ampules of Aroclor 1254, each of 50mg were considered acceptable for experimental procedures.

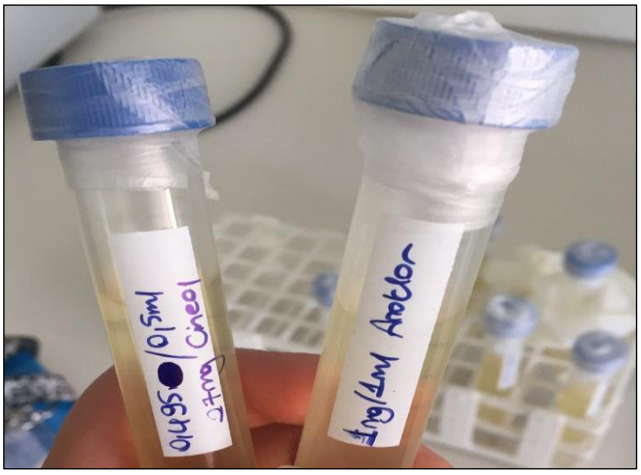

Figure 3.1. The falcon tubes containing the prepared solutions of Aroclor 1254 and 1,8 Cineole according to dosing protocol.

3.2. Animal and Ethics

A total of 32 male rats weighing 200-300g were used in the study. Experimental animals were fed free, at room temperature, %55-60 humidity, and 12 hours of dark/light environment. All experimental stages were conducted per the guidelines provided by the

Animal Experiments Ethics Committee, Faculty of Medicine, Pamukkale University. The Ethics Committee Approval number is PAUHADYEK-2022/28.

3.3. Selection and Categorizing of Animals

Thirty-two white albino rats weighing in the range of 200 g and 300 g were selected after approval from the Committee. The rats were then classified into four groups: the Control group, the Aroclor group, the Cineole group and the Aroclor + Cineol group. This is simplified in the following manner:

Table 3.1. Dose administration of Aroclor, 1,8 Cineole to four groups for thirty days

	GROUP	NUMBER OF RATS	DOSE	ROUTE OF ADMINISTRATION
1	Control	8	0.5 ml of corn oil	Oral gavage
2	Aroclor	8	1 ml (Aroclor;1 mg/kg)	Intraperitoneally (i.p.)
3	Cineole	8	0.5 ml (Cineole;100 mg/kg)	Oral gavage
4	Aroclor + Cineole	8	1 ml (Aroclor;1 mg/kg) + 0.5 ml (Cineole;100 mg/kg)	i.p. (Aroclor) + Oral gavage (Cineole)

3.4. Formation of Toxic Model in Rats

After arranging the groups, the rats being placed in standard room conditions and fed with regular diet were administered the respective drugs and toxicity-creating substances in doses as the control group was administered with corn oil (orally; 0.5-1ml), cineole group was administered for consecutive thirty days (orally; 100mg/kg), Aroclor was administered dose with a break of two days (total of ten doses given intraperitoneally; 1mg/kg) and Aroclor + Cineole group following the former group's dose protocol. The selected doses were managed according to the weight of the rats measured. Additionally, the weights of the rats were measured.

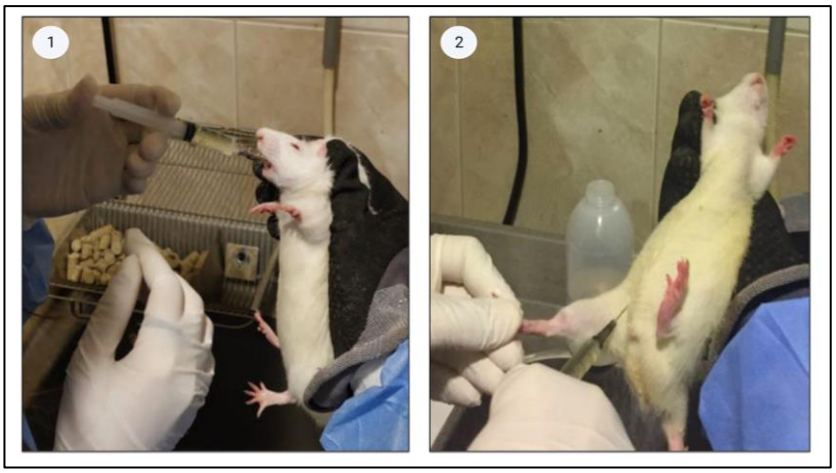

Figure 3.2 Administration of Selected Substances. 1. Administration of 1,8 Cineole and Corn oil orally. 2. Administration of Aroclor 1254 intraperitonially

Table 3.2. Thirty days protocol to administer 1,8 Cineole and Aroclor 1254

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
		1st Day Cineole	**2nd Day** Cineole & Aroclor	**3rd Day** Cineole	**4th Day** Cineole	**5th Day** Cineole & Aroclor
6th Day Cineole	**7th Day** Cineole	**8th Day** Cineole & Aroclor	**9th Day** Cineole	**10th Day** Cineole	**11th Day** Cineole & Aroclor	**12th Day** Cineole
13th Day Cineole	**14th Day** Cineole & Aroclor	**15th Day** Cineole	**16th Day** Cineole	**17th Day** Cineole & Aroclor	**18th Day** Cineole	**19th Day** Cineole
20th Day Cineole & Aroclor	**21st Day** Cineole	**22nd Day** Cineole	**23rd Day** Cineole & Aroclor	**24th Day** Cineole	**25th Day** Cineole	**26th Day** Cineole & Aroclor
27th Day Cineole	**28th Day** Cineole	**29th Day** Cineole & Aroclor	**30th Day** Cineole			

3.5. Anesthesia and Sacrifice of Animals

The anesthesia was carried out after 24 hours of the dosing protocol. For the anesthesia procedures, the anesthetic and sedative agents were Ketamine and Xylazine with respective doses of 87mg/kg and 13 mg/kg. Both agents were given in conjugation while the doses were adjusted in ml.

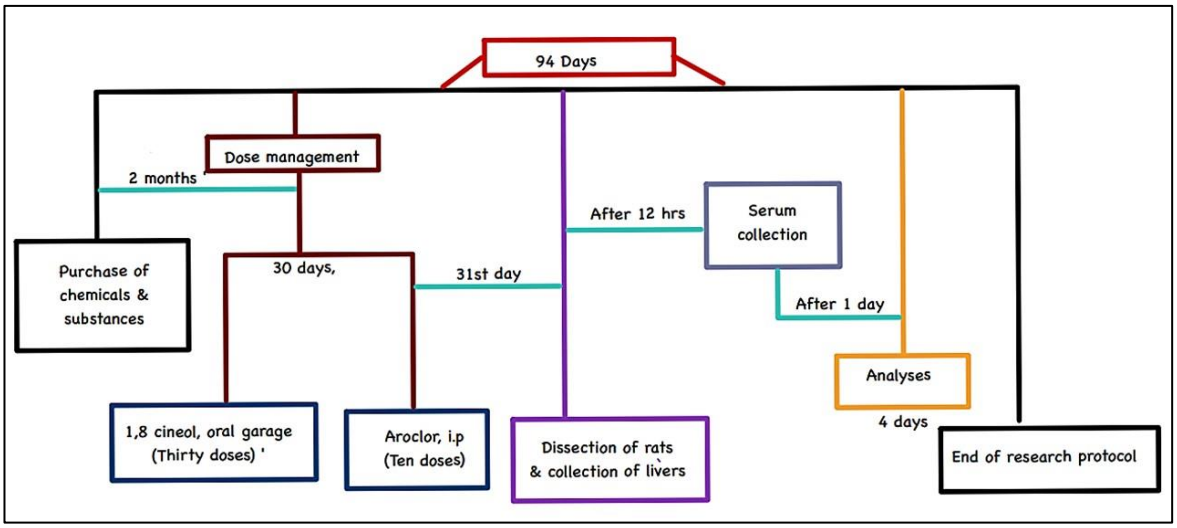

Figure 3.3. Schematical diagram to follow the research protocol

The sacrifice of rats was held by giving anesthesia and letting the animals in sedative mode for 15 to twenty minutes. After the loss of consciousness, the rats were finally dissected, taking blood near the heart as a first step after opening the abdomen region with scissors for dissection. The next step comprised taking out the liver.

Figure 3.4. Dissection of one of the 32 rats is being carried out

3.6. Histopathological Analysis

At the end of the study, liver samples of euthanized rats were collected and fixed in 10% buffered formaldehyde solution. Small sections were cut into the liver samples to ensure better penetration of the detection solution into the tissues. The next day, formaldehyde solutions were changed and kept in solution for two more days.

At the end of this period, liver samples were trimmed and placed in tissue tracking cassettes. The cassettes containing the tissue samples, which were kept in formaldehyde for another day, were placed in the basket of the fully automatic tissue tracking device (Leica ASP300S; Leica Microsystem, Nussloch, Germany) in the evening.

A routine tissue tracking procedure was performed on the device overnight. For routine tissue monitoring, liver samples were first transferred from 70% low-grade alcohols to 100% high-grade alcohols and the water in the tissues was removed. Following this process, they were passed through two separate xylol series in the device and the fats in the tissues were dissolved. All tissue cassettes were placed in hot paraffin to allow the hot paraffin to pass into the spaces formed by removing water and fat. After this process, the tissues were removed from the device and the blocking process was started.

For this purpose, liver samples, whose routine follow-up was completed, were taken into the tissue embedding device (Leica Histocore Arcadia H) (Leica Microsystem, Nussloch, Germany), which was heated the night before, and paraffin blocks were performed. After the blocking process, all blocks were taken to the cold table section of the tissue embedding device. The samples placed in special metal molds were kept on this tray for about 1.5 hours, allowing the hot paraffin to cool and freeze.

After cooling, each paraffin block separated from the metal molds was After cooling for 4-5 hours, 3 serial sections of 4-5 μm thickness were taken for the liver and 3 serial sections for the Leica 2155 rotary microtome (Leica Microsystem, Nussloch, Germany) for both histopathological and immunohistochemical examinations. A series of sections and two series of normal slides were drawn into polylysine slides. A series of sections taken from normal slides were stained with hematoxylin-eosin (HE), which is a routine pathology staining procedure, and examined under a light microscope. examined. For the staining procedure, the tissue sections taken on the slides were kept in the oven at 60°C for 2 hours to ensure that the paraffin melted, the sections dried, and completely adhered to the slides. To completely dissolve the remaining paraffin in the tissue sections, they were passed through 3 separate xylol series for 30 minutes each. After these procedures, the tissues were allowed to rehydrate by switching from high-grade alcohols to low-grade alcohols (100, 96, 90, 80 and 70% alcohols, respectively).

Thus, water-based hematoxylin and eosin staining of the sections was performed. For this purpose, routine hematoxylin-staining was done by staining in Harris hematoxylin solution for 15 minutes and immediately afterward in eosin solution for 3 minutes. HE staining was continued. Following this stage, the tissue sections were transferred from low-grade alcohols to high-grade alcohols (70, 80, 90, 96 and 100% alcohols, respectively), and the water in the tissues was completely removed. After these procedures, the polishing stage with xylol was started, a special adhesive, Entellan, was dropped on the sections, the coverslip was closed and examined under a microscope. Histopathological findings include hyperemia, edema, bleeding, and inflammatory cells were evaluated according to their infiltration.

3.7. Immunohistochemistry Method

For immunohistochemical evaluations, tissue sections taken on special polylysine slides were kept in an oven set at 45°C overnight before staining, ensuring that the sections adhered to the slide and dried completely. After drying, the slides were taken out of the oven the next morning and the staining procedure began. For this purpose, all sections were deparaffinized and rehydrated as detailed in the histopathological examination method. For immunohistochemical examination, sections were injected with streptoavidin- biotin complex peroxidase method was applied. Ready-to-use commercial kits were used for this purpose. Liver sections were detected with *Bcl-2* (Anti-Bcl-2 antibody (ab196495)) and *Bax* (Recombinant Anti-Bax antibody [E63] (ab32503) antibodies. All primary and secondary antibodies were obtained from Abcam (England) and used by diluting at 1/100 dilution. For the staining procedure, tissue sections were first melted in paraffin on xylene sections. Then, the sections were passed through graded alcohols, and the sections were rehydrated by giving them water. Then, it was washed in distilled water for 10 minutes.

To eliminate endogenous peroxidase activity on the sections, they were incubated with hydrogen peroxide prepared in 3% methanol for 20 minutes. Then in citrate buffer solution 2 times 5 boiled in the microwave oven for minutes. After the sections cooled down, they were washed twice in PBS for 10 minutes each. Then, they were incubated with normal serum for 45 minutes to eliminate nonspecific background staining was carried out. After this, primary sera were dropped onto the sections without washing and incubated in the refrigerator overnight. The next day, the sections taken from the refrigerator were washed in PBS for the same time and in the same manner, and then incubated with streptavidin for 30 minutes and washed twice in PBS for 10 minutes each. After this procedure, tissues were treated with biotin serum for 30 minutes. The sections were washed in the same way and stained with freshly prepared DAB (3,3'-diaminobenzidine) chromogen solution. Mouse and Rabbit Specific HRP/DAB IHC Detection Kit - Micro-polymer (ab236466) was used as the secondary kit and DAB was used as the chromogen. For negative controls, antibody diluents were replaced at the primary antibody stage. Counter-staining was performed with Harris hematoxylin, and

after this step, the coverslip-sealed preparations were examined under a light microscope to evaluate expressions.

3.8. Serum Collection

To collect the serum, the blood was stored in biochemical Tubes or EDTA tubes for 10 to 20 minutes at room temperature. The serums from all blood samples were then taken out, collected, and stored in Eppendorf tubes at -80 degrees. The tubes were then shifted for centrifugation at 5000 rpm for 10 minutes.

3.9. Tissue homogenization

For tissue homogenization, the protocol given by Next Advance was followed. The number of beads and buffer to give a specific sample size was according to - 1 volume/mass of tissue: 1 volume of beads: 2 volumes of buffer. The tissues were rinsed in ice-cold PBS to remove the excess blood thoroughly and weigh it before homogenization (Ph 7.4). Then the process was started by weighing the tissues first (ideally 300 mg was preferred). The second step was cutting them into smaller pieces to yield better results. The PBS solution added into the tubes was according to the weight of hepatic tissue at a ratio of 1: 2. The tubes containing the beads, tissue, and PBS were then subjected to the required homogenizer at 12 rpm for 2 minutes. The last step was conducted by centrifugation of homogenates for 5 minutes at 15000 rpm to get the supernatant.

3.10. Biochemical Analysis

Rat TAS and TOS ELISA, kits of BT LAB (Bioassay Technology Laboratory) were considered appropriate for experimental procedures. Following the serum storage and tissue homogenization at an appropriate temperature according to the protocol given,

the first step was reagent preparation (standards 1-5 and dilution of wash buffer) was carried out as instructed at room temperature.

The next step included determining the no. of strips required for assay and 50uL addition of standard to standard well. The samples in 40uL quantity were added to sample wells following the addition of 10uL anti-TOS antibody and anti-TAS antibody. The inclusion of 50uL of streptavidin-HRP in each sample well and standard well was conducted very carefully. Incubation at 37 degrees for 60 minutes was performed for both plates after covering the plates with each sealer.

After incubation, the plates taken out were washed five times with wash buffer, adding 50ul substrate solution A and B to each well of the two plates. Again the plates were incubated for 10 minutes at 37 degrees in the dark. The last step required a stop solution of 50ul addition to each well, which immediately changed the blue color to yellow.

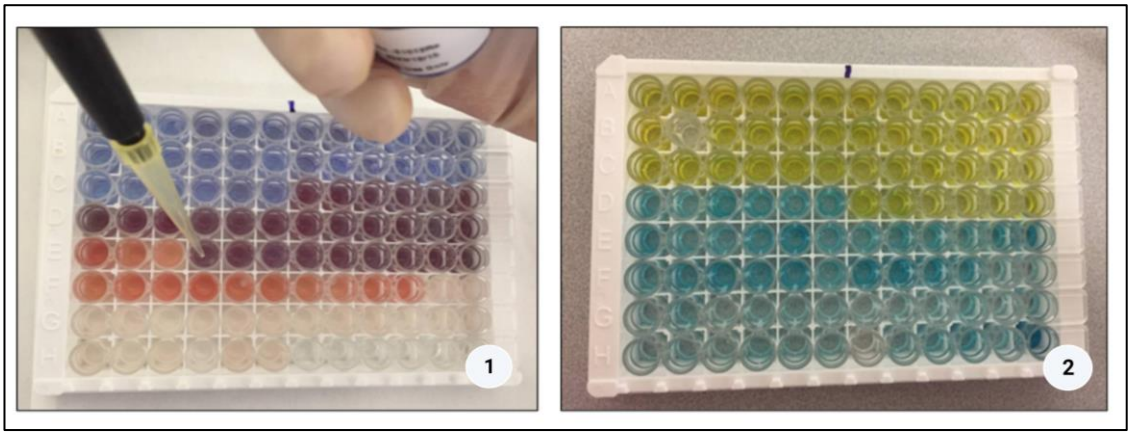

Figure 3.5. Steps followed to carry out biochemical analysis i.e. TOS and TAS following ELISA protocol. 1. Inclusion of streptavidin-HRP in each sample well. 2. Addition of stop solution to each well.

Optical density was measured using a microplate reader (name of the reader) setting wavelength at 450nm. A standard curve was plotted by allotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis. (A general picture of how the graph should be plotted).

3.11. Molecular Analysis

3.11.1. RNA isolation

The protocol of a one-step RNA reagent by Bio Basic for extraction of RNA from hepatic tissues was followed and carried out. First, the tissues were homogenized by putting the samples in 1 ml of ONE STEP-RNA reagent per 50-100 mg of liver tissue.

The samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Later, 200ul of chloroform was added to each microcentrifuge tube. The incubation was then again carried out for 2-3 minutes after shaking the tubes for 15 seconds. This was further proceeded by centrifugation at 12000xg for 15 minutes at 4 degrees. The last step involved the addition of phenol which eventually separated into a lower phenol-chloroform phase, cloudy white interphase, and a colorless upper aqueous phase.

After transferring the aqueous phase to a fresh tube, 500ul of isopropyl alcohol, according to the protocol, was mixed in the aqueous phase. The incubation at room temperature was carried out for 10 minutes, and then tubes were shifted for centrifugation process at 11000xg for 15 minutes at 4 degrees temperature. The RNA precipitate was visibly seen as a gel-like pellet on the side or bottom of the tube.

This step was carried out by washing RNA pellets with 75% ethanol in the required amount as described by the protocol. The samples were mixed with ethanol (500ml) by vortexing and centrifugation for 5 minutes at 8800 rpm.

30 µl of RNase-DNase-free water was added to the pellet and pipetted. Tubes wrapped with parafilm were stored at -20ºC until the concentration and purity of the isolated RNA were measured.

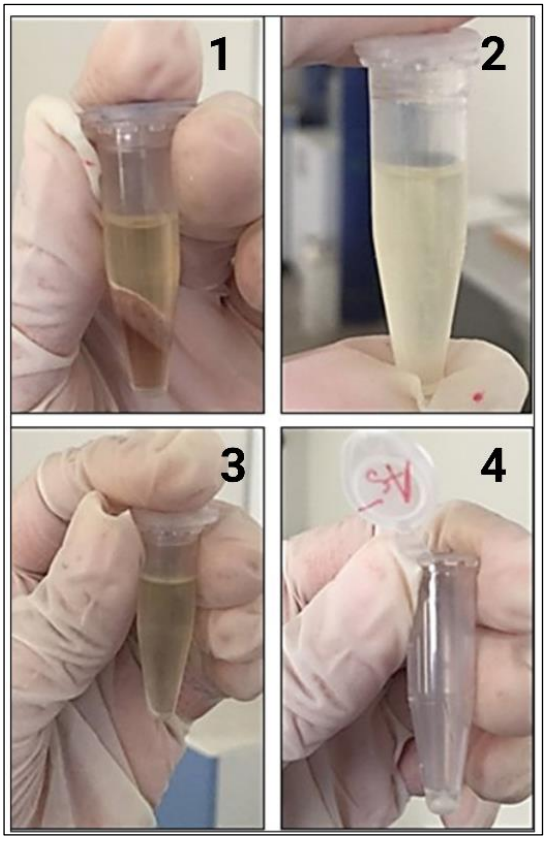

Figure 3.6. Steps involved in RNA isolation. 1. Centrifugation after incubation to separate DNA, proteins, and RNA. 2. Addition of chloroform to form a cloudy white interphase. 3. Addition of isopropyl alcohol. 4. Separation of aqueous phase from RNA pellet after centrifugation.

RNA quantification was done by using the Biotech Take 3 Microvolume Plates protocol. Different samples up to 2uL were put at the same time on a plate and put into an Epoch reader for microvolume absorbance and fluorescence quantitation. Intuitive interphase was applied by the take 3 reader for the management of data analysis and acquisition. RNA reading protocol was selected, and blank and sample sections were selected by setting up the plate. Following this Read blank button and Read sample button were clicked. The result was later obtained in the form of raw and background subtracted absorbance data. The data of the calculated concentrations were finally extracted.

3.11.2. cDNA synthesis

cDNA synthesis from the isolated RNAs was carried out using the One Script Plus cDNA synthesis kit in accordance with the manufacturer's protocol.

Table 3.3. Components with the required volume necessary for cDNA synthesis.

COMPONENTS	VOLUME
5X RT Buffer	4 µl
dNTP	1 µl
Primer	1 µl
Total RNA	2µg
One Script Plus RTase	1 µl
Nuclease-free water	Up to 20 µl

The mixtures prepared for cDNA synthesis were completed with RNase DNase-free water to a final volume of 20 µl. After incubating for 15 minutes at 55°C in the Thermal Cycler device, it was kept at 85°C for 5 minutes to stop the reaction. Synthesized cDNAs were stored at -20°C to perform Real-Time PCR was preserved.

Figure 3.7. Mixtures for cDNA synthesis incubated in a Thermal Cycle device

3.11.3. RT-PCR analysis

Step One Plus RT-PCR device, which can read a 96-well microplate, was used for RT-PCR analysis. Sequences of genes involved in oxidative stress, inflammation, and cell death were determined on a custom basis. Primer sequences are shown in Table 3.4. B-actin was used as a reference housekeeping gene. RT- PCR; BlasTaq 2X qPCR MasterMix kit from synthesized RNA samples was carried out following the manufacturer's protocol.

Table 3.4. Primer sequences with their length while B-actin as reference housekeeping gene observed by RT-PCR.

		PRIMER SEQUENCE	LENGTH (BP)
β-actin	Forward	TGACAGGATGCAGAAGGAGA	**20**
	Reverse	TAGAGCCACCAATCCACACA	**20**
Bcl-2	Forward	CCGGGAGATCGTGATGAAGT	**20**
	Reverse	ATCCCAGCCTCCGTTATCCT	**20**
Bax	Forward	GTGGTTGCCCTCTTCTACTTTG	**22**
	Reverse	CACAAAGATGGTCACTGTCTGC	**22**
TNF-α	Forward	AAATGGGCTCCCTCTCATCATCAGTTC	**27**
	Reverse	TCTGCTTGGTGGTTTGCTACGAC	**23**
IFN-γ	Forward	CACGCCGCGTCTTGGT	**16**
	Reverse	TCTAGGCTTTCAATGAGTGTGCC	**23**
IL-1β	Forward	CACCTCTCAAGCAGAGCACAG	**21**
	Reverse	GGGTTCCATGGTGAAGTCAAC	**21**
COX-2	Forward	AAGGGAGTCTGGAACATTGTGAAC	**24**
	Reverse	CAAATGTGATCTGGACGTCAACA	**23**
iNOS	Forward	GCATCCCAAGTACGAGTGGT	**20**
	Reverse	GAAGTCTCGGACTCCAATCTC	**21**

The mixture prepared according to the number of samples was distributed into a 96-well microplate and cDNA samples were added to the wells (Table 3.5). Then into a 96-well microplate. Primers were distributed respectively. RNase-DNase-free solution

with a total volume of 20 µl for each well. The reaction was added with water and the 96-well microplate was covered with sealing film.

Table 3.5. Mixing protocol for RT-PCR

COMPONENTS	VOLUME
BlasTaq 2X qPCR	10 µl
Forward primer	0.5 µl
Reverse Primer	0.5 µl
Template DNA	1 µl
Nuclease-free water	Up to 20 µl

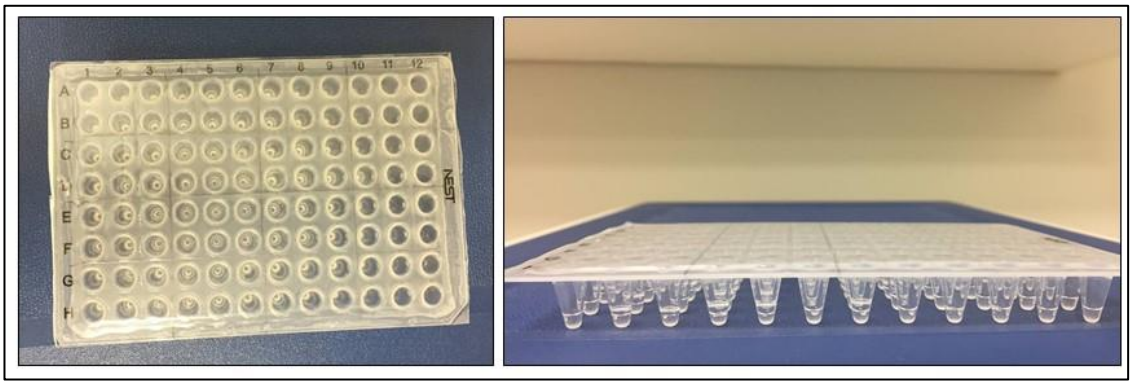

Figure 3.8. 96 well plate upper and side view, ready to be put in the RT-PCR machine.

The control group used in the comparison in the analysis of the data belonged to the Aroclor group, Cineole group, and Aroclor + Cineole group and the results were evaluated using the Gene Globe Data Analysis (Qiagen) program. The reaction protocol of RT-PCR is given in the form of Table 3.6.

Table 3.6. Reaction protocol for RT-PCR.

Stage	Heat	Duration	Cycle
Enzyme Activation	95° C	3 min	1
Denaturation	95° C	15 sec	40
Attachment/ Extension	60° C	1 min	40

3.12. Statistical Analysis

Statistical analysis of histopathological and immunohistochemical scores in the study One-way ANOVA test was done in the SPSS 22.00 package program. Differences between groups were determined and compared by the Duncan test. Analysis of RT-PCR data using the 2'ΔΔCT method and quantitation was done with a computer program. On the Internet-based Gene Globe platform Volcano Plot in the 'RT² Profiler™ PCR Array Data Analysis' program, analyses were used. It was done using Two-way ANOVA and post-hoc Tukey in GraphPad Prism (version 7) program. Values of $p < 0.05$ were considered statistically significant.

4. RESULTS

4.1. Results of Body Weight

Change in body weight for thirty days drug and toxic substance is shown in Figure 4.1. The graphical representation shows that the mean body weight Change in the Aroclor group increased as compared to the Control. While the mean body weight of the Aroclor-Cineole group decreased in contrast with the Aroclor group. There is no statistically significant difference in body weight change between the groups.

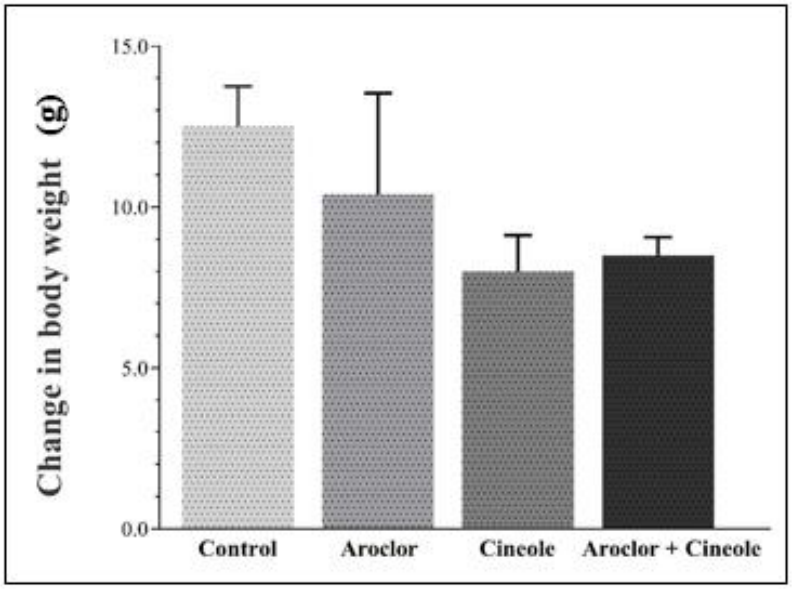

Figure 4.1. Change in body weight for rats

4.2. Histopathological Results

Histopathological results of liver sections are observed in Figure 4.2. The groups appeared normal in the histopathological examination of liver sections according to groups, control, and cineole. In the Aroclor group, there was hyperemia in the veins and swelling around the veins. More prominently, edema was noted. In this group, most lymphocytes and inflammatory cell infiltration, including neutrophils and leukocytes, were detected. Degenerative changes in hepatocytes were also noted in this group. Cineole treatment was observed showing that the pathological findings improved.

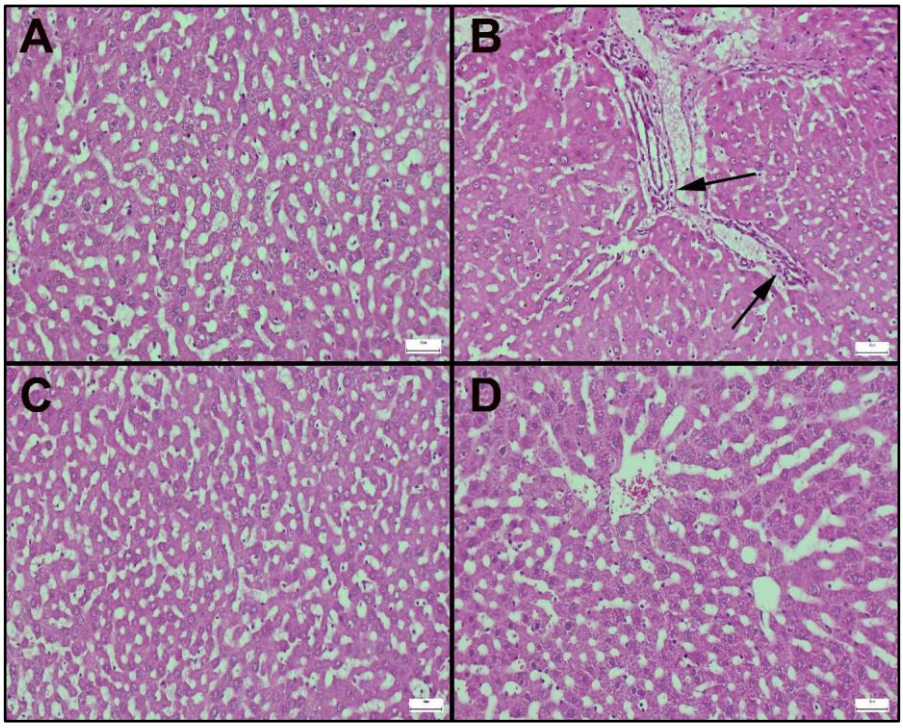

Figure 4.2. Histopathological results of liver sections; A. Control B. Aroclor C. Cineole D. Aroclor + Cineole. Histopathological appearances of livers according to groups. (A) Appearance of normal liver in the control group. (B) Swelling and disruption of regular structure and inflammatory cell infiltrates in hepatocytes in the Aroclor group (arrows), (C) Significant improvement in pathological findings in the Aroclor + Cineole group. (D) Normal liver histological appearance in the Cineole group, HE, Bars=50µm.

4.3. Immunohistochemical Results

Immunohistochemically results of liver sections are observed in Figure 4.3 and Figure 4.4. Additionally, immunohistochemical scores are displayed in Table 4.1. In the examination of Bax staining, immunohistochemically stained sections of the liver, very mild or negative expressions were observed in the control and cineole groups, while increased expressions were observed in the Aroclor group. Expressions were found to decrease in the Aroclor + Cineole group. In Bcl-2 staining, there was a significant positive reaction in the control and cineole groups. While observed, it was noted that the expressions decreased in the Aroclor group. An increase in expression was observed in the Aroclor + Cineole group.

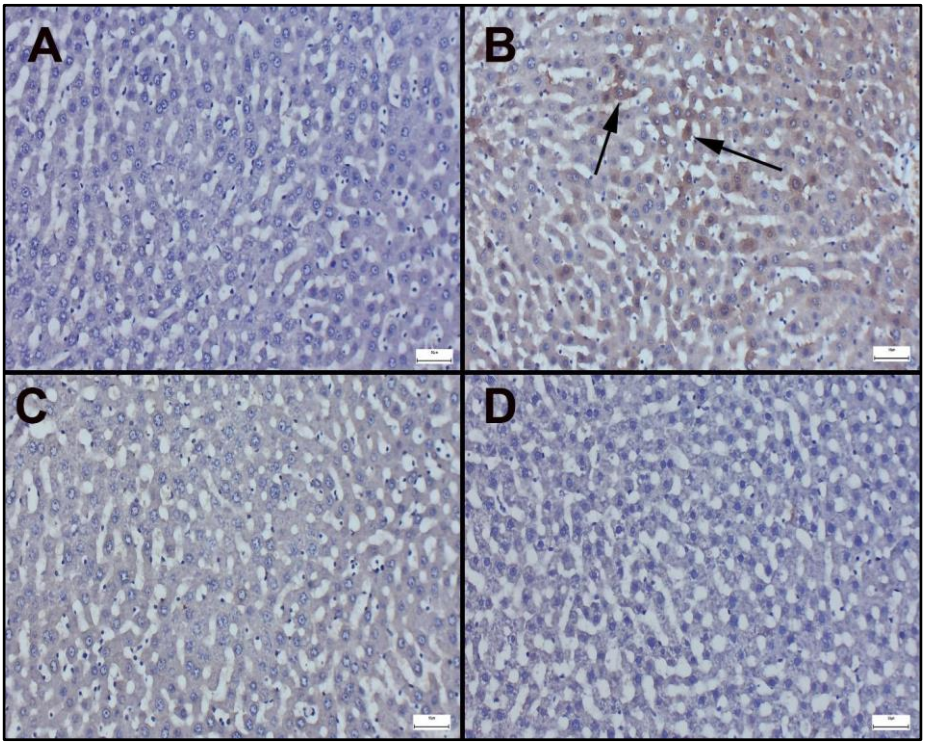

Figure 4.3. Bax immunohistochemical results of liver sections; (A. Control B. Aroclor C. Cineole D. Aroclor + Cineole). (A) Negative expression in the control group. (B) Increase in expressions in the Aroclor group (arrows), (C) Decrease in expressions in the Aroclor + Cineole group. (D) Negative expression in the Cineole group, Streptavidin biotin peroxidase method, Bars=50µm.

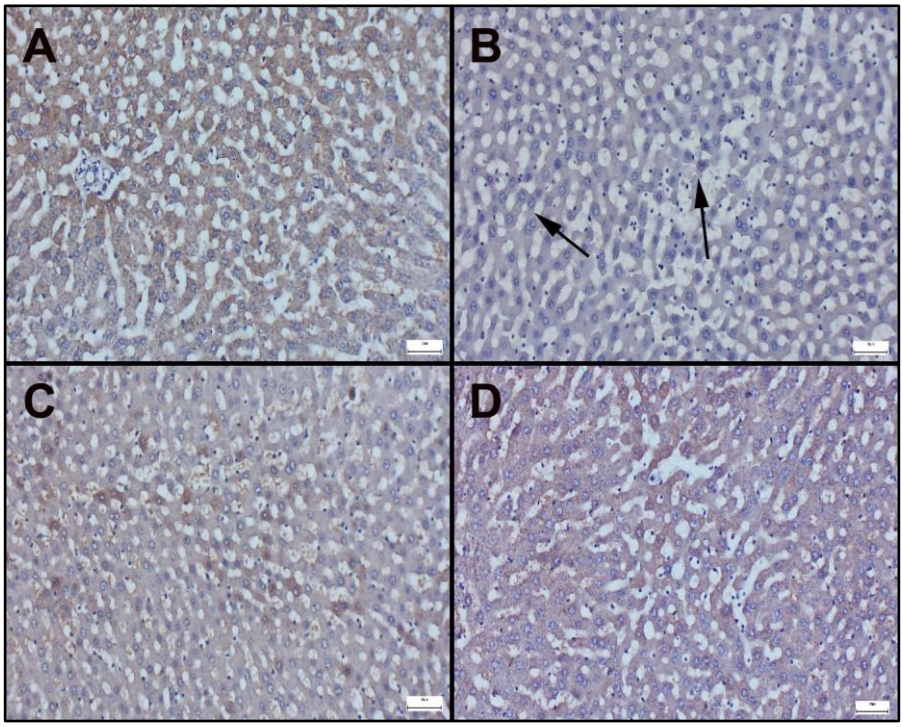

Figure 4.4. Bcl-2 immunohistochemical results of liver sections; A. Control B. Aroclor C. Cineole D. Aroclor + Cineole. (A) Significant expression in the control group. (B) Significantly decreased expressions (arrows) in the Aroclor group, (C) Increased expressions in the Aroclor + Cineole group. (D) Significant expression in the Cineole group, Streptavidin biotin peroxidase method, Bars = 50µm.

Table 4.1 Statistical analysis of immunohistochemical scores

	Bax	**Bcl-2**
Control	0.14 ± 0.37^{a}	2.42 ± 0.53^{a}
Aroclor	2.28 ± 0.48^{b}	0.71 ± 0.48^{b}
Cineole	0.14 ± 0.37^{a}	2.57 ± 0.53^{a}
Aroclor + Cineole	1.14 ± 0.69^{c}	1.85 ± 0.37^{c}
p value	< 0.001	< 0.001

Data are given as mean ± standard deviation.
Differences between groups indicated by different superscripts in the same column are statistically significant.

4.4. Biochemical Results

Changes in TAS and TOS levels associated with oxidative damage in blood serum are given in Figure 4.5. TAS levels decreased significantly and TOS levels increased significantly in the Aroclor-applied group compared to the control group (p<0.05). In the Aroclor + Cineol and Cineol groups, TAS levels increased significantly while TOS levels decreased significantly compared to the Aroclor group (p<0.05).

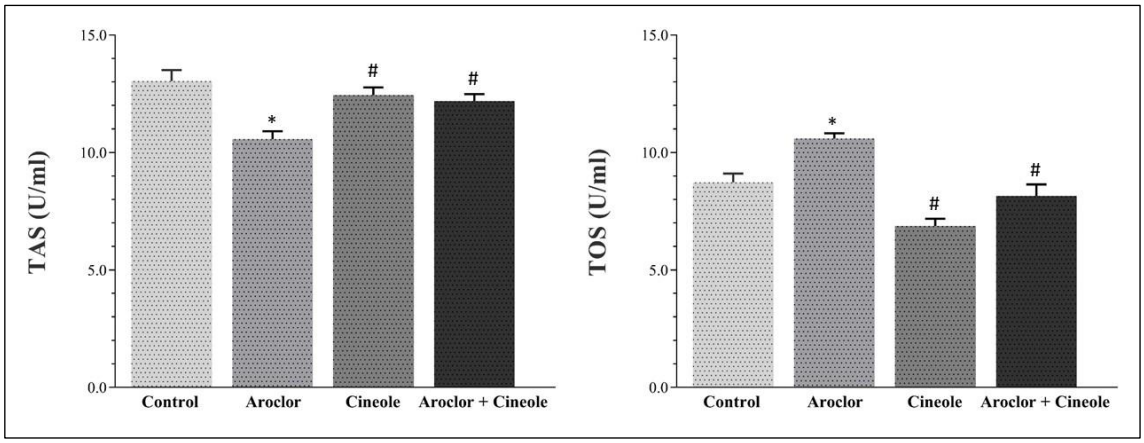

Figure 4.5. Changes in TAS and TOS levels in blood serum
*: Compared to the control group; #: Compared to Aroclor group. p<0.05

Changes in TAS and TOS levels associated with oxidative damage in liver tissue are given in Figure 4.6. TAS levels decreased significantly, and TOS levels increased significantly in the Aroclor-applied group compared to the control group (p<0.05). In the Aroclor + Cineol and Cineol groups, TAS levels increased significantly while TOS levels decreased significantly compared to the Aroclor group. (p<0.05).

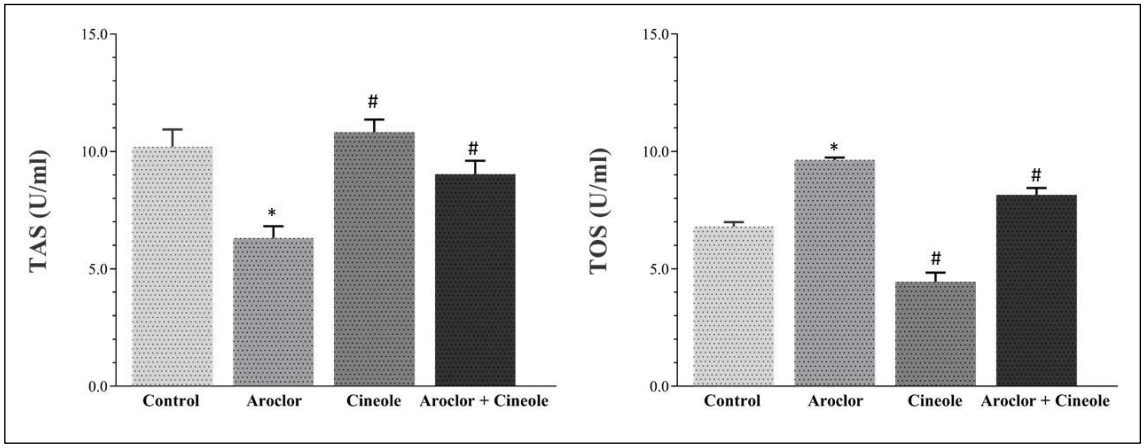

Figure 4.6. Changes in TAS and TOS levels in liver tissue
*: Compared to the control group; #: Compared to Aroclor group. $p<0.05$

4.5. Molecular Analysis Results

Gene expression findings related to apoptosis, inflammation, and oxidative damage in liver tissues are given in Table 4.2. The fold changes of mRNA expression of Aroclor, Cineole, and A1254+1,8C groups were Compared with the control group.

Table 4.2. Gene expression findings

Gene	Control		Aroclor		Cineole		Aroclor + Cineole	
	Fold change (Fc)	*p*	Fold change (Fc)	*p*	Fold Change (Fc)	*p*	Fold change (Fc)	*p*
Bax	1.00	-	3.63	0.0026*	-1.37	0.3526	1.96	0.002*
Bcl-2	1.00	-	-1.24	0.22	-1.03	0.7843	1.08	0.5779
TNFα	1.00	-	7.31	0.0023*	-1.03	0.9109	3.09	0.0075*
COX-2	1.00	-	5.81	0.017*	1.09	0.5908	3.95	0.0004*
IFN-γ	1.00	-	2.20	0.0119*	-1.06	0.4454	1.85	0.0045*
iNOS	1.00	-	2.61	0.0088*	-1.14	0.4426	1.97	0.038*
IL-1β	1.00	-	1.97	0.045*	-1.09	0.3786	1.55	0.0032*

*According to Control group ; p <0.05 was considered statistically significant.
Fc: Fold changes; Stimulation (+) and suppression (-)

It was determined that *Bax, TNFα, COX-2, IFN-γ,* and *iNOS* mRNA expressions were increased by *3.63, 7.31, 5.81, 2.20,* and *2.61* times respectively, in the Aroclor group

compared to the control group ($p < 0.05$). It was determined that *BCl-2* mRNA expression decreased -*1.24*-fold in the Aroclor group compared to the control. No significant change was observed in *Bax*, *Bcl-2, TNFα, COX2, IFNγ, iNOS,* and *IL-1β* mRNA expressions in the Cineole group compared to the control group ($p > 0.05$). On the other hand, it was determined that administration of Aroclor and Cineole simultaneously increased *Bax*, *Bcl-2, TNFα, COX2, IFNγ, iNOS,* and *IL-1β* mRNA expressions by *1.96*, *1.08*, *3.09*, *3.95*, *1.85*, *1.97*, *1.55* times, respectively, compared to the control.

5. DISCUSSION

This thesis study aims to investigate the therapeutic effects of Cineole against Aroclor-induced liver toxicity in rats and the oxidative damage, inflammation, and apoptotic cell death pathways that play a possible role in its mechanism of action. In this regard, using the ELISA method following the experimental protocol; Histopathological analysis by HE staining, immunohistochemical evaluation of *Bax* and *Bcl-2* scores, biochemical detection of TAS and TOS levels by ELISA method, and molecularly *Bax, Bcl-2, TNF-α, IL-1β, COX-2, IFN-γ, iNOS* gene expressions by RT-PCR method.

There are different possible mechanisms through which PCBs can alter normal functioning and homeostasis of the body, causing toxicity and elevated oxidative stress in different organs. PCBs as being persistent and ubiquitous environmental pollutants have long-term hepatic chronic effects. A1254 (PCBs)-mediated oxidative stress has been researched because of its significant toxicological impact. In one of the studies, it was stated that A1254-induced cytotoxicity, mitochondrial dysfunction in isolated rat hepatocytes, and in vitro genotoxicity risk assessment in rat liver and human liver subcellular fractions (Aly & Domènech, 2009; Obach et al., 2008). In the present study of hepatotoxicity, the toxic effect of A1254 was investigated on rats' hepatic organs to characterize the molecular mechanisms involved in hepatotoxicity, focusing on the role played by oxidative stress.

Furthermore, deformation in the hepatic structural integrity is one of the signs of PCB toxicity. In one of the recent studies, histological examination revealed central and peripheral necrosis, destruction of lobular architecture, and the creation of septa with sinusoidal dilatation due to significant liver injury (Sekaran et al., 2012).

The histopathological evaluation carried out in our research revealed that Aroclor caused morphological dysfunctionality in hepatocytes. Hyperemia, swelling around the veins, edema, and inflammatory cell infiltration were significantly observed. However, Cineol treatment improved these pathological findings. We can further hypothesize that Cineol with its antioxidant ability lessens the structural integrity of hepatocytes badly affected by the toxicity of Aroclor.

Other than that, the immunohistochemistry *Bax* and *Bcl*-2 score data supported that the damage was caused by Aroclor to liver cells and tissues. The administration of Cineol considerably showed a protective effect towards Aroclor-induced damage by lowering the Bax levels statistically. Based on these findings, it is possible to conclude that apoptotic cell death plays a part in the Aroclor harm process. Cineole, on the other hand, has been shown in the literature to have anti-apoptotic action.

Several cross-sectional human population studies imply that POPs bioaccumulation contributes to the development of pre-diabetes and type 2 diabetes. Some epidemiologic findings indicate that individuals who are exposed to POPs are significantly more likely to develop obesity-related metabolic dysfunction, such as insulin resistance, hyperinsulinemia, diabetes mellitus, and cardiovascular disease (Chen et al., 2008; Grandjean et al., 2011). One study found that prolonged Aroclor 1254 exposure affected glucose homeostasis. Insulin levels rose, followed by substantial increases in serum resistin, *TNF-α*, and *IL-6*, demonstrating that Aroclor 1254-induced obesity was related to insulin resistance. The increase in blood glucose levels might have been caused mostly by IR signal pathway inhibition in skeletal muscle and liver. These findings should aid in understanding the development of obesity and type 2 diabetes induced by PCBs (Gerich et al., 1973; Zhang et al., 2015).

In our study, an increase in weight was observed in Aroclor given group. This result supports the theories and observations given in literature that an increase in insulin resistance followed by IR signaling pathway inhibition in skeletal muscle and liver can lead to an increase in body weight caused by PCBs. However, when Cineole was administered to rats exposed to Aroclor toxicity, a decrease in body weight was observed. This finding aids in understanding a potential concept that Cineol can potentially inhibit glucose homeostasis by recovering IR signal pathway as well as decreasing pro-inflammatory cytokines to lower insulin resistance induced by PCBs.

Oxidative stress occurs when the ratio of prooxidants to antioxidants is out of balance, resulting in the buildup of oxidatively damaged molecules. According to several studies, increased cellular oxidative stress is a fundamental underlying mechanism of

PCB-mediated cell activation and dysfunction. As a result, the toxic effect of Aroclor 1254 on hepatocytes may be due to increased lipid peroxidation, as demonstrated in studies, which was based on the critical role of lipids in maintaining membrane

structure and function, and ultimately cellular viability, and ROS production, which induces oxidative stress (Banudevi et al., 2005; Sridhar et al., 2004). ROS formation can be caused by the production of HO.- and O2.-ions at the abrasion site, leading to lesser GSH activity and the accumulation of HO.- and O2.-ions. As a result, there is evidence of a significant increase in H2O2 and MDA in the hepatocytes of A1254-treated rats (Sekaran et al., 2012).

Aroclor 1254 was shown to produce ROS in rat testicular mitochondria and impair spermatogenesis. Although mitochondria are most likely the primary source of ROS in most cells, nothing is known regarding ROS production in hepatocyte mitochondria (Venkataraman et al., 2008). Mitochondria hold antioxidant ability, under physiological conditions decreasing the generation of ROS, production, and removal of already generated ROS. Another possibility for increased cellular toxicity can be the impairment of the antioxidant activity of mitochondria in hepatocytes leading to alternation in intrinsic membrane properties, DNA damage, and eventually cell death. The inhibition of the mitochondrial respiratory chain and mitochondrial Beta-oxidation by A1254 can also be a skeptical target for mitochondrial dysfunction. Conditions that support enhanced mitochondrial synthesis of reactive oxygen species (e.g., high Ca2+ concentrations or defective mitochondrial antioxidant defense systems) may cause MPT and cell death (Aly & Domènech, 2009; Martins et al., 2008).

Another hypothesized mechanism for Aroclor 1254-induced ROS formation and lipid peroxidation is the stimulation of cytochrome P450 enzymes, which results in the release of ROS. Aroclor 1254 has been shown to increase hepatic ethoxy-, methoxy-, and pentoxyresorufin O-deethylase (EROD, MROD, and PROD, respectively) activities in rat liver. A recent study found that PCB-induced oxidative stress in rat kidneys might be attributed to CYP1A1 upregulation. In addition, PCB-induced hepatic CYP450 (total CYP450, CYP1A, and CYP1B). As a result, it is fair to speculate that the CYP system is involved in Aroclor 1254-induced ROS generation in hepatocyte mitochondria. Increased ROS production would cause permanent damage to mitochondrial membrane lipids and proteins, leading to mitochondrial malfunction and, eventually, cell death (Bonfanti et al., 2009; Lu et al., 2009).

The antioxidant capabilities of 1,8-cineole are critical to its therapeutic potential since it is excellent at neutralizing ROS and improving cellular defense systems. ROS buildup causes oxidative stress, associated with the development and progression of many

clinical illnesses, including inflammation, aging, and neurodegenerative diseases (Reuter et al., 2010). In some in vitro experiments, 1,8-cineole has shown significant free radical scavenging activity, demonstrating its potential to neutralize ROS and protect cells from oxidative damage (Ryu et al., 2014). Treatment with 1,8-cineole increased antioxidant enzymes activities such as *SOD* and *CAT* increased total antioxidant capacity, and decreased ROS and *MDA* content in grass carp hepatocytes, effectively suppressing oxidative stress induced by di(2-ethylhexyl) phthalate (Cui et al., 2020).

Several models, including RAW264.7 cells and ankle tissues, have shown comparable antioxidant properties (Yin et al., 2020). In these investigations, 1,8-cineole was found to increase antioxidant enzyme activity while decreasing ROS levels, thereby relieving the oxidative stress caused by monosodium urate. In chickens, co-administration of 1,8-cineole with BPA considerably boosted the activities of antioxidant enzymes such as *SOD*, *GPx,* and *CAT* while decreasing high levels of oxidative parameters such as *MDA*, *iNOS,* and *NO* (Liu et al., 2021).

1,8-cineole has been shown to prevent LP. In research on 1,8-cineole's protective impact against LP in rat liver microsomes, 1,8-cineole successfully prevented the development of Thiobarbituric acid reactive chemicals, which are routinely used indicators of LP (Hsouna et al., 2019). This research suggests that 1,8-cineole can protect biological components from oxidative damage, such as membranes and lipids.

1,8-cineole's antioxidant action is achieved in part by activating the *Nrf2*/*Keap1* pathway, which is a key regulator of cellular protective responses to inflammation and oxidative stress (He et al., 2020). The treatment with 1,8-cineole elevated the nuclear translocation of *Nrf2,* a transcription factor that links to antioxidant response elements in the promoter regions of targeted genes, resulting in higher levels of phase II detoxifying enzymes and antioxidant proteins like heme oxygenase-1 and NAD(P)H: quinone oxidoreductase 1 (NOQ1) (Venkataraman et al., 2023). Additional research has shown that 1,8-cineole protects liver cells and pheochromocytoma cells by activating the *Nrf2*/*Keap1* pathway and enhancing *SOD* Expression (Chen et al., 2022; Z. Jiang et al., 2019). Treatment with 1,8-cineole has been demonstrated in several cellular and tissue models to increase the activities of antioxidant enzymes such as *SOD, GPx*, and *CAT* while decreasing the levels of oxidative parameters created by diverse ROS inducers such as LPS, hydrogen peroxide, and cigarette smoke (Di et al., 2022; Mirghaed et al., 2018).

According to the research done, the TAS and TOS levels in serum and liver tissues concerning the Aroclor group significantly increased and decreased. Moreover, Cineole administered to the Aroclor group as compared to solely Aroclor, lowered the TOS level remarkably. This particularly justifies the antioxidant activity of Cineol. These results particularly give grounds for the studies carried out for evaluating the oxidative stress of Aroclor and the antioxidant properties of Cineol. Aroclor can cause hepatotoxicity by initiating oxidative stress through different cellular mechanisms and hindering the antioxidative mechanisms. On the other hand, Cineol possesses a strong antioxidant property by obstructing the excessive oxidative stress mechanisms by bringing ROS levels to an optimum range.

Apoptosis is an active cellular process of gene-directed self-destruction in which cells die in a regulated manner, either spontaneously or in reaction to certain environmental stimuli or substances (Das et al., 2009). Because the liver is the primary location for the detoxification of hazardous metabolites, it is thought to be a possible target for PCB-induced adverse consequences. Apoptosis has been linked to oxidative stress and ROS in numerous cell types, including hepatic cells (Kasahara et al., 2002). The *Bcl-2* family serves critical functions in the apoptosis of cells. *Bcl-2* family proteins govern the activation of the internal mitochondrial-dependent apoptotic pathway. *Bax* translocation from the cytoplasm to the mitochondria triggers cytochrome c release, increasing cell death. *Bcl-2*, on the other hand, is essential for preventing the initiation of the mitochondrial apoptotic pathways (Mohamad et al., 2005; Yao et al., 2012).

Bcl-2 and *Bax* expression is involved in the balance of pro-apoptotic and anti-apoptotic signals for cytochrome c release from mitochondria into the cytosol. However, increased *Bcl-2* expression and decreased *Bax* expression may cause resistance to apoptotic cell death in damaged cells. Therefore, decreasing *Bcl-2* and increasing the expression level of *Bax* can eliminate damaged cells by inducing apoptotic cell death (Ahagh et al., 2019; Kumar et al., 2018).

Through anti-apoptotic and pro-apoptotic actions, 1,8-cineole has shown potential therapeutic implications. On the one hand, 1,8-cineole has been proven in numerous models to be capable of preventing apoptosis and boosting cell survival. For example, It has been discovered that 1,8-cineole inhibits retinal apoptosis in diabetic eyes by lowering the expression of pro-apoptotic markers *(Bax)* and boosting the expression of anti-apoptotic proteins (*Bcl-2*) (Kim et al., 2020). Furthermore, 1,8-cineole inhibited

isoprenaline-induced apoptosis by reducing the *Bax/Bcl-2* ratio and cleaved caspase-3 expression in both H9c2 cardiomyocytes and rat heart tissue (Wang, Zhen, et al., 2021). Co-administration of 1,8-cineole and BPA led to a significant reduction in apoptosis-positive cells, as well as a decrease in mRNA and protein levels of pro-apoptotic genes (C*aspase-3, Bax, and p53*) stimulated by BPA, while promoting the expression of the anti-apoptotic gene *Bcl-2* (Liu et al., 2021). Furthermore, 1, 8-cineole may have prevented △ψm loss in ISO-induced cardiomyocyte damage. In vitro and in vivo studies on apoptosis-related proteins confirmed that 1, 8-cineole might reduce the *Bax/Bcl-2* ratio (Wang, Zhang, et al., 2021).

In our study, it was found that Cineole was able to significantly decrease the fold change of apoptosis regulator *Bax* protein when administered to Aroclor-damaged hepatocytes. Additionally, the decrease in *Bcl-2* expression levels in the Aroclor group compared to the control indicates that hepatic cells are one of the primary targets for apoptotic cell death and initiation of carcinogenesis. This finding can support the statement that Cineole can be a potential agent against the imprudent apoptosis initiated by Aroclor or PCBs by inhibiting the *Bax* protein upregulation and increasing the anti-apoptotic *Bcl-2* protein activity to halter carcinogenesis.

Pollutants in the environment have been demonstrated to contribute to the development of inflammatory reactions through a variety of methods. Endothelial cells are activated by AhR agonists such as coplanar PCBs and TCDD (Hennig et al., 2002; Toborek et al., 1995). A recent study revealed that interleukin-1-like cytokines, particularly *TNF-α,* may facilitate dioxin's hepatotoxic effects, such as induction of hepatocyte suicide or apoptosis (Pande et al., 2005). *TNF-α* belonging to transmembrane II protein with an intracellular N terminus possesses signaling potential either as an integrated membrane protein or acting as a cytokine after proteolytic cleavage (Hehlgans & Pfeffer, 2005; Locksley et al., 2001).

TNF-α is important in the following activation of a very complicated biological cascade involving chemokines, cytokines, and endothelial adhesions that attracts and activates neutrophils, macrophages, and lymphocytes at sites of injury and infection. TNF-α is a kind of cytokine that increases in response to oxidative stress. This factor can be generated as pre-inflammatory cytokines during liver inflammation (Q. Chen et al., 2017). Additionally, recent research has shown that *TNF- α* gene expression in the liver is enhanced in cases of liver injury. Several research studies have found that chemical

agent-induced liver damage is structurally linked to inflammation and significantly raises TNF- α levels (Ahmadi-Naji et al., 2017; Karimi-Khouzani et al., 2017).

In one recent research, PCB in various doses influenced the relative expression of *IL-1β, IL-8*, and *TNF-α* gene mRNA following zebrafish exposure for 1, 7, and 14 days which could lead to liver tissue structure, and increase the liver index (Qamar et al., 2022). It ought to be additionally noted that PCBs 101, 153, and 180 increase *TNF-α* and start apoptosis in macrophages by activating mitochondrial pathways (Ferrante et al., 2011). It was also evaluated in one of the current studies that the concomitant rise in *TNF- α* with decreased insulin action may potentially generate HPTA aberrations. This might be related to PCBs stimulating AhR (Crofton & Zoeller, 2005).

Pro-inflammatory cytokines are small proteins released by immune cells that play a crucial role in mediating and regulating body inflammation. The dysregulation of these cytokines' production leads to both the onset and progression of a variety of inflammatory disorders. Venkataraman et al. used a dextran sodium sulfate (DSS)-induced rat model of colitis to scrutinize the anti-inflammatory effects of 1,8-cineole on colonic inflammation (Venkataraman et al., 2023). 1,8-cineole effectively decreased colonic shortening, MPO enzyme activity, and pro-inflammatory cytokine levels, including interleukin *(IL) 6, IL-1β, TNF-α, and IL-17A* (Venkataraman et al., 2023). Furthermore, in an acute pancreatitis mouse model, 1,8-cineole administration resulted in lower cytokine levels of *TNF-α, IL-1β,* and *IL-6*, reflecting the results found with thalidomide, a *TNF-α* inhibitor. Notably, 1,8-cineole therapy raised the anti-inflammatory cytokine *IL-10* (Lima et al., 2013). Bastos et al. discovered that inhaling 1,8-cineole decreased tracheal contractions, lowered *TNF-α* and *IL-1β* levels, and suppressed eosinophil and neutrophil aggregation in bronchoalveolar lavage fluid (BALF) specimens in a guinea pig model of ovalbumin (OVA)-induced asthma (Bastos et al., 2011).

In the comparison of gene expression of *TNF-α* between control and Aroclor, Aroclor was able to elevate the *TNF-α* expression in hepatocytes at a significant level. It could suggest that Aroclor by upregulation of AhR receptors increases the ROS that can lead to the activation of macrophages to initiate apoptosis at the damaged site. The other possibility can be the mitochondrial dysfunctionality causing an increase in proinflammatory cytokines levels. On the other hand, Cineole treatment towards hepatocytes damaged by Aroclor lowered the *TNF-α* proposing the anti-inflammatory

effect of Cineole by regulating AhR protein to lessen Aroclor toxicity as stated in different studies.

IL-1β is a powerful pro-inflammatory cytokine that plays an important role in host defensive responses to infection and injury (Dinarello, 1996). It is also the most well-known and researched of the 11 IL-1 family members. It is generated and released by a range of cell types, although the great bulk of research has focused on its synthesis by innate immune system cells such as monocytes and macrophages (Takeuchi & Akira, 2010). It is created as an inactive 31kDa precursor known as pro-IL-1β in response to molecular motifs carried by pathogens known as PAMPs. PAMPs affect gene expression pathways by acting on macrophage pattern recognition receptors (PRRs). Caspase-1, a pro-inflammatory protease, cleaves pro-IL-1β (Thornberry et al., 1992). Inflammasome activation is mediated by the liver's ROS. It has been shown in a study that macrophages released pro-IL-1, which is triggered by neutrophil-derived proteinases, in a mouse model. The interaction between neutrophils and macrophages advances *IL-1β* maturation and results in IL-1β-driven inflammation in the IR liver (Sadatomo et al., 2017). Highlighting the inflammation caused by PCBs, Many reports have found that PCB exposure increases the production of *TNF-α* or interleukins (*IL-1β, IL-6, or IL-12*), indicating activation of AhR (Baker et al., 2013; Crofton & Zoeller, 2005), stimulation of inflammatory pathways (Hennig et al., 2002), activation of the *NF- κβ* (Kwon et al., 2002), or inhibition of the AMPK pathways (Ferrante et al., 2014).

1,8 cineol exhibits potent anti-inflammatory and antioxidant properties. Talking about the effect of 1,8-cineol on *IL-1β, TNF-α, and IL-1β* levels that govern airway mucus hypersecretion in asthma was substantially reduced by 1,8-cineol (Pries et al., 2023). Furthermore, 1,8 cineol was able to reduce the *TNF*-α and *IL-1β* levels which were elevated by LPS-induced acute Pulmonary Inflammation in Mice (Uwe R Juergens et al., 2004). Additionally, it was reported that 1,8 cineol was able to reduce the production of *TNF-α, IL-1β, IL-6, and IL-8* in monocytes by 60%. The same effect was observed in the production of *TNF-α, IL-1β, IL-4*, and *IL-5* (Yun Li et al., 2016). Additionally, 1,8 cineol was able to reduce the vascular endothelial inflammation interceded by pro-inflammatory cytokines in LPS-induced systemic inflammation in rat models (F. Jiang et al., 2019). Moreover, lowered the *TNF-α* and *IL-1β* mRNA expression in human umbilical vein endothelial cells in vitro (Kutlu et al., 2021).

In our results, there was not a drastic change between fold changes of *IL-1β* in the group of rats given Aroclor solely and the rat group administered with Aroclor and Cineole together. The Aroclor didn't increase the *IL-1β* drastically. This can either be due to the limitation of data being put in or inflammation caused by Aroclor can be independent of *IL-1β* induced inflammation pathway. However, more research is needed to carry out whether the release of the pro-inflammatory cytokines is mediated by AhR or *NF- κβ* pathway concerning dose adjustment of PCBs.

Furthermore, DNA damage is associated with inflammatory cell infiltration and increased *COX-2* protein expression. However, it's still in debate whether *COX-2* protein activation is elevated by the *NF-κβ* pathway or ER stress via the *XBP1*/*IRE1α* pathway. A recent study suggested that All A1254 exposure groups were associated with increased IRE1 and XBP1 protein expression. As a result, we postulated that following low-level PCB exposure, *COX-2* expression could be controlled by the IRE1-XBP1 route rather than the *NF- κβ* pathway. Additional study is needed to ascertain if the effects of A1254 being exposed to *COX-2* are directly correlated with IRE1-XBP1 activation, such as either the presence or absence of XBP1 or inhibiting IRE1-XBP1 using inhibitors (Aly & Domènech, 2009; Sudarshan et al., 2010; Zhang et al., 2014).

Highlighting the effect of 1,8 cineol on *COX-2*, In HaCaT cells, 1,8-cineole was discovered to inhibit UVB-induced *COX-2* protein and mRNA synthesis. The inhibition of the AhR resulted in lower *COX-2* expression and phosphorylation of ERK1/2 in these cells. On top of that, topical administration of 1,8-cineole to the mouse skin successfully delayed tumor development and reduced the growth of tumors while inhibiting *COX-2* expression in vivo (Essaghir et al., 2010). The suppression of *COX-2* by 1,8-cineole may help to reduce inflammation by lowering *NF-κβ* expression.

Our results showed a drastic increase in *COX-2* gene expression in the group of Aroclor. However, while comparing the fold change of *COX-2* expression between the Aroclor group and Cineole administered to Aroclor damaged group, the possible reason that can happen is the excessive hematological damage caused by Aroclor in liver tissues that even Cineole was unable to lower the *COX-2*-dependent inflammation pathway.

IFN-γ is a cytokine that is involved in both protective immunological responses and immunopathologic processes. Certain transformed cells can be induced apoptosis by *IFN-γ* via traditional signaling methods involving the production of activated STAT1

homodimeric complexes. This pathway was discovered to result in the IFN/STAT1-dependent activation of genes encoding intracellular or membrane components that cause cellular death, such as *caspase-1* (IL-1I converting enzyme or ICE) (Chin et al., 1997; Detjen et al., 2001), or *Fas* and *FasL* (Xu et al., 1998). According to one of the studies, there was not a significant rise in the *INF-γ* in the 20 μg PCB-treated group but the rise was highly visible with the increase in concentration of PCBs (40 μg) (Ahmed et al., 2018). Concurrently with the present study, PCBs 52 and 77 increase the amount of in the *INF-γ* mouse thymocyte cultures (Sandal et al., 2005). In addition, female C57BL/6J x FVB mice exposed to TCDD produced more *IFN-γ* (Van Esterik et al., 2015).

The anti-inflammatory effect of Cineol towards Aroclor toxicity seems to be a pre-eminent factor from the results we have obtained. Lowering *IFN-γ* levels in liver toxicity can be a new step towards finding out how Cineol lowers the pro-inflammatory cytokines through different cellular mechanisms. However, it's also debatable to dig deeper to find out cellular mechanisms through which Cineol or other terpenes can provide protective mechanisms against inflammation caused by PCBs or Aroclor.

In pathological situations, the inducible form of *iNOS* is expressed in liver cells. NO, as a by-product of the oxidation of L-arginine and citrulline plays an important role in the hepatic physiology as well as pathophysiology (Iwakiri & Kim, 2015). This is done by the action of three isoforms of NO synthases named neuronal NOS (nNOS or NOS1), inducible *NOS* (*iNOS* or *NOS2*), and endothelial *NOS* (*eNOS* or *NOS3*). NO produced by *iNOS* is associated with the etiology of liver inflammation and diseases (Iwakiri & Kim, 2015). Although all isoforms are present in hepatic tissue, the most significant are *iNOS* and *eNOS*. *iNOS* is found in macrophages and can be activated during proinflammatory processes (Clemens, 1998; Lowenstein et al., 1992). It is also found in endothelial cells, hepatocytes, HSCs, and cholangiocytes (Curran et al., 1990; Helyar et al., 1994; Rockey et al., 1996). Because *iNOS* generates far more NO than any other isoform and lacks precise control, it may cause hepatic tissue damage mostly by nitrosylation of thiol residues. Furthermore, NO damage is exacerbated by oxidative stress because NO and O2 mix to form peroxynitrite, a highly reactive radical capable of disrupting various cellular processes. Nitrosative stress is caused by an excess of RNS production and disrupts various biological activities (Dröge, 2002; Loughran et al., 2017; Torok & Physiology, 2016).

Macrophages are phagocytic cells that capture and digest foreign particles, debris, and pathogens to preserve tissue homeostasis and promote tissue healing. 1,8-cineole inhibited LPS-induced NO generation in mouse macrophage cell lines and aided acute lung damage in a mouse model (Kim et al., 2015; Zhao et al., 2014). It has also been stated that 1,8 cineol taken from Achillea millefolium L. essential oil decreased the level of NO and down-regulate *iNOS, TNF-α, and IL-6* by suppressing the inflammatory responses mediated by lipopolysaccharide-stimulated RAW 264.7 macrophages (Chou et al., 2013). It has also been stated that 1,8 cineol by repressing the *NF-κβ* pathway diminished the *iNOS*-derived NO overexpression while retrieving the *eNOS*-derived NO to the optimum level in HUVECs (Linghu et al., 2016a).

These data agree swith our results of oxidative damage in liver tissues concerning related research and studies. A significant increase of *iNOS* levels in the Aroclor group suggests that Aroclor exposure to tissues induces oxidative damage by the production of RNS via Nitrative stress. However, a decrease in *iNOS* levels in Cineol agrees with the theory that either Cineol possesses an ameliorative effect towards the oxidative damage in liver cells by Aroclor either by suppressing the inflammatory response caused by lipopolysaccharide-stimulated macrophages or by hindering the *NF-κβ* pathway to lower down the *iNOS*-derived *NO* overexpression. This result can hypothesize that 1,8 cineol can inhibit the oxidative damage of Aroclor by depressing the *NO* overexpression.

6. CONCLUSION

In this thesis study, exposure to Aroclor 1254 has been observed to induce liver toxicity. Subsequent administration of 1,8 Cineole was found to mitigate oxidative stress, reduce inflammatory damage, and ameliorate histopathological injuries in liver cells. Additionally, it was determined that 1,8 Cineole exerts its protective effects through anti-apoptotic mechanisms. Nevertheless, further investigations are required to elucidate the detailed molecular mechanisms of 1,8 Cineole in conferring protection against PCBs or Aroclor 1254 toxicity.

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9. APPENDICES

Appendix-1 Ethics Committee approval