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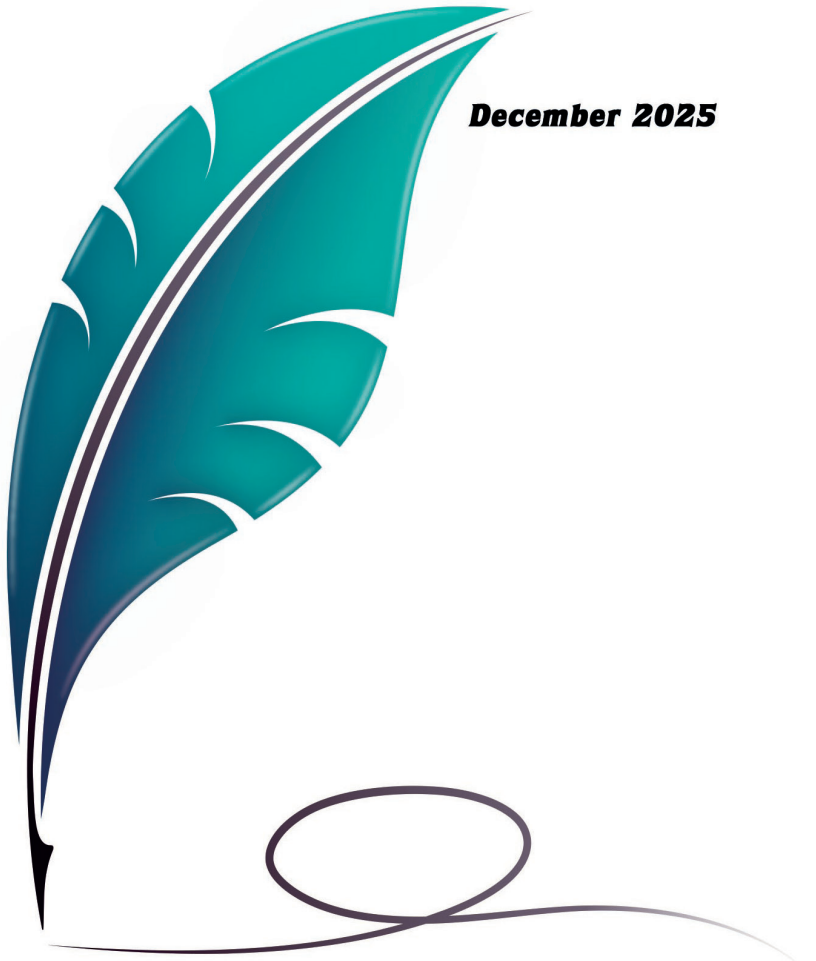

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Editor

Prof. Dr. Hasan Akgül

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Chapter
1

AN OVERVIEW OF THE GENOTOXIC EFFECTS OF PESTICIDES

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1. INTRODUCTION

Pesticides, which have become indispensable inputs of modern agriculture, are used to protect plants, enhance yield, and ensure food security. Millions of tonnes of pesticides are applied in agricultural production worldwide each year, a significant portion of which enters environmental cycles. As biologically active chemicals, it is unsurprising that pesticides exert toxic effects not only on target organisms but also on non-target species (Sari, 2022). In particular, the genotoxic potential of pesticides has been extensively investigated under occupational exposure conditions and chronic low-dose environmental exposure, with numerous studies indicating that these substances can damage DNA integrity (Sari, 2022; Karsli et al., 2022; Ili & Sari, 2024; Yılmaz et al., 2025).

Genotoxicity refers to all forms of damage occurring directly or indirectly to genetic material and is assessed using biomarkers such as DNA strand breaks, base modifications, chromosomal aberrations, micronucleus formation, mutations, and recombination changes. Genotoxic effects arising from pesticide exposure represent a critical risk in both acute and chronic exposure contexts, impacting cell proliferation, apoptotic processes, and long-term cancer development (Kapeleka et al., 2021; Sánchez-Alarcón et al., 2021). This risk extends not only to humans but also to aquatic organisms, soil organisms, and the broader ecosystem.

Therefore, investigating the genotoxic effects of pesticides is crucial not only from a toxicological standpoint but also for public health, environmental health, and sustainable agricultural policy. This chapter provides an overview of the genotoxic effects of pesticides and the biological mechanisms involved, and discusses future perspectives on their implications for human and environmental health.

2. GENOTOXICITY AND ASSESSMENT METHODS

Genotoxicity refers to the effects of chemical and physical agents capable of causing direct or indirect damage to the DNA, the genetic material of living organisms (Phillips & Arlt, 2009). DNA damage can manifest in various forms, including single- and double-strand breaks, DNA–DNA or DNA–protein cross-links, adduct formation, chromosomal aberrations, and

micronucleus formation (Phillips & Arlt, 2009; Sezginer & Dane, 2021). When intracellular repair mechanisms are insufficient, such genetic alterations may lead to mutations, cell death, or oncogenesis, causing serious biological consequences at both the cellular and organismal levels (Phillips & Arlt, 2009).

Pesticides can induce base modifications, strand breaks, sister chromatid exchanges, micronucleus formation, and chromosomal breaks (Bolognesi & Morasso, 2000). A range of laboratory- and field-based methods have been developed to assess pesticide-induced genotoxicity. Among the most widely used are the comet assay (single-cell gel electrophoresis), the micronucleus test, chromosomal aberration assays, and oxidative damage markers. The comet assay is a highly sensitive method that measures DNA strand breaks at the single-cell level. Numerous biomonitoring studies among agricultural workers have reported a strong association between pesticide exposure and increased DNA damage (Bhalli et al., 2006; Bolognesi et al., 2011). The micronucleus assay is based on the formation of micronuclei when chromosomal fragments or whole chromosomes are excluded from the main nucleus during cell division. This test is widely used to detect cytogenetic damage due to pesticide exposure (Bolognesi et al., 2011). Chromosomal aberration tests are suitable for evaluating chromosomal breaks, translocations, and other structural abnormalities (Sherif et al., 2023), while oxidative DNA damage products such as 8-OHdG are used to assess the indirect genotoxic effects of pesticides (Koureas et al., 2014). Since each method provides different levels of information, many studies employ them together or complementarily.

3. PESTICIDES AND THEIR GENOTOXIC POTENTIAL

Pesticides are chemical and biological substances widely used in agriculture to control harmful organisms that reduce plant yield and quality (Pimentel, 2005). In agriculture, insecticides target insect pests, herbicides target weeds, and fungicides target fungi causing plant diseases (Aktar et al., 2009). Pesticides are applied not only in the field but also post-harvest to reduce losses caused by insects and fungi in stored products (Arthur, 1996). In public health, pesticides are an important tool for controlling vector-borne diseases such as malaria, particularly through mosquito control (van den Berg et al., 2012). In forest ecosystems, controlled pesticide applications are used to limit economic and ecological losses caused by tree pests (Leroy, 2025).

Pesticides are also widely used in urban and domestic settings for pest control (Meftaul et al., 2020), and in veterinary practice, derivatives are employed to manage internal and external parasites in farm and companion animals (Küntüz et al., 2023). However, excessive and indiscriminate use of pesticides can lead to environmental pollution, biodiversity loss, and adverse effects on human health (Carvalho, 2017). Consequently, integrated pest management approaches aimed at reducing pesticide use and minimising environmental impact are increasingly emphasised (Pretty & Bharucha, 2015).

Pesticides are classified according to their chemical structures and target organisms. The most common classes include organophosphates, organochlorines, carbamates, pyrethroids, neonicotinoids, herbicides, and fungicides. Organophosphate pesticides are among the most widely used agricultural chemicals worldwide. Beyond their well-known acute effects via acetylcholinesterase inhibition, there is substantial evidence for DNA damage caused by these agents. Chlorpyrifos, malathion, and diazinon exert genotoxic effects through oxidative stress and mitochondrial dysfunction (Boussabbeh et al., 2016; Montanarí et al., 2024). Organochlorines such as DDT, aldrin, and dieldrin accumulate in adipose tissue due to their lipophilic nature and have long half-lives (Jayaraj et al., 2017). They possess high genotoxic potential due to endocrine disruption, increased reactive oxygen species, and DNA binding tendencies (Jayaraj et al., 2017; Dwivedi et al., 2022). Carbamates, including carbaryl and carbofuran, have been associated with DNA strand breaks and increased micronuclei. Their genotoxicity is often mediated by oxidative stress and metabolite reactivity (Sharma & Sharma, 2012; Saquib et al., 2021). Broad-spectrum synthetic pyrethroids such as deltamethrin, permethrin, etofenprox, and tau-fluvalinate are generally considered to have low acute toxicity; however, some studies indicate that they can lead to DNA damage and chromosomal aberrations (Sari, 2022; Ili, 2024; Ili & Sari, 2024; Yılmaz et al., 2025). Neonicotinoids such as imidacloprid and thiamethoxam have been subjects of recent debate, with evidence of genetic damage in both humans and bees (Gauthier et al., 2018; Guo et al., 2020). Herbicides and fungicides have also been extensively evaluated for genotoxic risk; for instance, bentazone and chloridazon have been shown to increase DNA damage and chromosomal aberrations in earthworm cells (Ulukütük & Ciğerci, 2020).

The genotoxic potential of pesticides arises through multiple biological mechanisms. Hence, the genotoxic profile depends not only on

chemical structure but also on metabolism, target tissues, and exposure duration. Oxidative stress is the most common mediator of genotoxicity. Many pesticides disrupt intracellular redox balance, impair mitochondrial function, and increase reactive oxygen species production (Čermak et al., 2018). Accumulation of reactive oxygen species results in base damage, single- and double-strand breaks, and replication stress in DNA (Khatib et al., 2022; Sule et al., 2022). Some organophosphates and fungicides interact directly with DNA, causing covalent binding, DNA–protein crosslinks, and adduct formation. This disrupts cell cycle control and increases mutation rates (Khatib et al., 2022). Growing evidence indicates that pesticides suppress DNA repair pathways such as nucleotide excision repair, base excision repair, and homologous recombination (Kaur & Kaur, 2018). This accumulation of DNA damage increases the risk of cellular ageing, death, or malignant transformation. Micronucleus formation, chromosomal aberrations, sister chromatid exchange, and aneuploidy are commonly used biomarkers of pesticide exposure. Given the diversity of genotoxic mechanisms, pesticide genotoxic potential is highly variable.

4. PESTICIDE-INDUCED GENOTOXICITY IN HUMANS

Occupational groups with intensive exposure to pesticides, such as agricultural workers, pesticide applicators, and greenhouse employees, constitute the population at highest risk for genotoxic effects. The potential of pesticides to damage genetic material in humans has been demonstrated in numerous biomonitoring studies using various genotoxic markers (Bull et al., 2006; Costa et al., 2006). A series of epidemiological and biomonitoring studies have shown that occupational pesticide exposure is significantly associated with DNA damage, chromosomal aberrations, sister chromatid exchanges, and increased micronucleus frequency. For instance, one study reported that individuals with intensive occupational exposure to pesticides exhibited increases in chromosomal aberrations, sister chromatid exchanges, and micronucleus frequency, with these effects becoming more pronounced as exposure duration or intensity increased (Bolognesi, 2003). In Turkey, a study on greenhouse workers exposed to pesticides reported significant genotoxic indicators in buccal mucosa cells, with micronucleus formation, karyolysis, and similar cytogenetic abnormalities being markedly higher in the exposed group compared to controls (Çelik et al., 2025). Another study examining agricultural pesticide exposure found that peripheral blood

lymphocytes of farmers had significantly higher frequencies of chromosomal aberrations relative to a control group. These findings collectively support the potential of pesticides to damage genetic material in humans within the context of occupational exposure (Demirhan et al., 2019). Furthermore, systematic reviews indicate that certain genetic polymorphisms may modulate susceptibility to pesticide-induced genotoxicity, with some individuals exhibiting heightened sensitivity due to variants in xenobiotic-metabolising genes (Pinto et al., 2024). These studies underscore that pesticide-induced genotoxicity arises not from singular mechanisms but through complex interactions of genetic and metabolic factors.

Pesticides disperse from agricultural sites into water, soil, and air, entering the food chain (Sarker et al., 2024). Such environmental dissemination leads to chronic low-dose exposure in the general population, creating an insidious, cumulative genotoxic risk. Particularly, lipophilic pesticides with slow degradation rates are prone to bioaccumulation. Analyses of food samples often detect multiple pesticide residues, indicating that individuals may be simultaneously exposed to both single active ingredients and compound mixtures. Some of these residues include hormone-disrupting and potentially carcinogenic compounds, highlighting the necessity of monitoring even low-dose, chronic dietary pesticide exposure for public health protection. Indirect genotoxic risks of environmental pesticide exposure may have especially severe health consequences for vulnerable populations, including children and pregnant individuals.

Certain demographic groups are considered more sensitive to the genotoxic effects of pesticides (Anguiano-Vega et al., 2020; Liu et al., 2024). Children, foetuses, pregnant individuals, and the elderly face heightened risk due to physiological and metabolic differences. High cell division rates and incompletely matured DNA repair mechanisms in children increase vulnerability to genotoxic agents, with organophosphates and pyrethroids particularly associated with oxidative stress and DNA damage in this population. During pregnancy, pesticides can cross the placental barrier, reaching the foetus and potentially causing genetic damage in developing tissues. Evidence from both animal and human studies suggests that prenatal pesticide exposure may adversely affect genomic integrity. In older individuals, age-related declines in DNA repair capacity and weakened

antioxidant defences can render pesticide-induced genotoxic damage more persistent.

5. PESTICIDE-INDUCED GENOTOXICITY IN ECOSYSTEMS

The ecological effects of pesticides are not limited to acute toxicity; they also produce long-term, often irreversible consequences by causing cumulative damage to the genetic material of organisms. Ecological genotoxicity refers to the potential of pesticides to induce DNA damage, chromosomal abnormalities, mutations, and genetic instability in all biological components of the environment. Such damage is not confined to individual organisms but can propagate to population levels and ultimately affect entire ecosystem processes. The persistence of pesticides in environmental matrices such as water, soil, and air renders ecological genotoxicity a widespread and enduring risk.

Aquatic ecosystems are among the primary environments through which pesticides are transported and dispersed (de Souza et al., 2020; Sari, 2022). Through rainfall, surface runoff, wind transport, and agricultural drainage, numerous pesticides reach aquatic habitats, becoming sources of genotoxic stress for fish, invertebrates, and amphibians. Studies in aquatic systems have shown that pesticide exposure can severely compromise DNA integrity in fish (Ergenler & Turan, 2023). The wide gill surface area, high metabolic activity, and lipophilic chemical absorption of fish amplify genotoxic effects. Observed outcomes include DNA strand breaks, elevated micronucleus frequency, chromosomal alterations in haematopoietic tissues, and increased oxidative stress markers, indicating direct destructive effects of pesticides on aquatic genomes. Such genetic disturbances lead not only to individual health issues but also to reduced reproductive success, lower juvenile survival rates, and eventual population declines.

Aquatic invertebrates are also highly sensitive to pesticides. As foundational components of many food webs, genetic damage in these organisms can propagate through the ecosystem. Pyrethroid and organophosphate pesticides have been shown to induce DNA strand breaks, developmental abnormalities, and disrupted gene expression in aquatic invertebrates (Sari, 2022; Shoaib & Ali, 2022). Such genetic harm can diminish invertebrate populations and disrupt the balance of aquatic ecosystems.

Amphibians are even more critically affected, as their dual reliance on aquatic and terrestrial environments and high developmental sensitivity render them vulnerable to environmental chemicals. Pesticide-exposed amphibians exhibit DNA damage, delayed metamorphosis, morphological deformities, and reduced reproductive success (Bridges, 2000; Roza et al., 2024).

Soil ecosystems serve as both repositories and active sites of pesticide impact. Considering the persistence of many pesticides in soil for months or even years, soil organisms experience continuous genotoxic pressure. For example, herbicides such as bentazone and chloridazon have been found to cause significant DNA damage in earthworms, which are key indicators of soil health (Ulukütük & Ciğerci, 2020). Observed effects include chromatin condensation, DNA strand breaks, mitotic irregularities, and increased apoptosis. Disruption of earthworm genetic integrity can affect essential soil processes such as organic matter turnover, aeration, humus formation, and nutrient cycling, creating cascading effects on agricultural productivity and soil ecosystem health.

Soil microorganisms are similarly sensitive, with chemical exposure increasing mutation rates, disturbing DNA synthesis, and dysregulating stress response genes (Vischetti et al., 2020). Declines in microbial diversity directly reduce soil fertility and impair ecosystem functions. Interference with nitrogen fixation, carbon cycling, and organic matter biotransformation by pesticides can diminish the long-term biological productivity of soils (Sim et al., 2022). The fact that these effects arise at the genetic level highlights the possibility of long-term, transgenerational impacts on ecosystem health.

Plants are not exempt from genotoxic effects. Pesticide use can induce mitotic irregularities, slowed DNA replication, and chromosomal anomalies in dividing meristematic cells, particularly in root tips (Ili & Sari, 2024). Genomic damage in plants reduces stress tolerance, leading to decreased yield, developmental defects, and compromised environmental adaptability. Evidence that pesticide-induced damage can be transmitted to subsequent generations suggests that genotoxicity may contribute to long-term genetic instability in agricultural production.

Bioaccumulation and biomagnification of pesticides across the food chain further enhance ecological genotoxicity (Mieiro et al., 2023; Saha &

Dutta, 2024; Tison et al., 2024). Lipophilic compounds, particularly organochlorines and some pyrethroids, accumulate in tissues and magnify toxicity in higher trophic levels, including birds, mammals, and predatory fish. Evidence from studies on these organisms highlights serious genotoxic consequences, including DNA damage, reproductive disorders, developmental irregularities, and embryonic loss. Population-level declines resulting from pesticide-induced genetic damage pose serious threats to ecosystem structural integrity.

Ecological genotoxicity is thus critical not only for individual organisms but also for the long-term sustainability of entire ecosystems. Genetic diversity is a fundamental determinant of ecosystem resilience, and pesticide-induced DNA damage reduces this diversity, weakening populations' capacity to adapt to environmental changes. Declines in genetic diversity can precipitate population collapses, local extinctions, and disruption of ecosystem services. Consequently, pesticides must be evaluated not only as agricultural productivity tools but also as environmental stressors threatening genetic and biological integrity.

Biomarkers and molecular techniques are essential for assessing ecological genotoxicity (Ladeira & Smajdova, 2017). Methods such as comet assays, micronucleus analysis, chromosomal aberration tests, gene expression profiling, and measurements of DNA repair enzyme activity are widely used to monitor environmental impacts. These approaches allow for early detection of pesticide-induced genetic damage and integration into environmental risk assessments (Azqueta & Collins, 2013).

The effects of pesticides on ecological genotoxicity are extensive, multi-layered, and long-term. DNA damage observed in both aquatic and terrestrial ecosystems threatens not only short-term organismal health but also population stability, biodiversity, and ecosystem functions. Genotoxicity must therefore be a core parameter in evaluating the environmental impact of pesticide use.

6. CONCLUSION

Current scientific evidence indicates that pesticides—particularly organophosphates, organochlorines, carbamates, pyrethroids, neonicotinoids, herbicides, and fungicides—pose significant genotoxic risks

to both humans and ecosystems. Laboratory studies, human biomonitoring data, and environmental observations collectively demonstrate that pesticide exposure is associated with increases in DNA strand breaks, micronucleus formation, chromosomal aberrations, and genetic instability. Over the long term, such genetic damage may contribute to mutation accumulation, cancer development, reproductive disorders, and heritable genetic problems.

The extent and manifestation of pesticide genotoxicity depend on multiple variables, including pesticide type, dose, duration and route of exposure, chemical composition, individual genetic susceptibility, and environmental conditions. Heterogeneity among studies indicates that some investigations report marked genotoxic effects, while others show no significant differences, reflecting that pesticide genotoxicity is a dynamic process shaped by exposure conditions and biological context.

Pesticide genotoxic effects are not limited to direct human exposure; they also occur indirectly through environmental contamination, the food chain, bioaccumulation, and biomagnification, leading to long-term genetic and ecological damage in aquatic and terrestrial ecosystems. Harm to non-target organisms, reductions in biodiversity, and disruptions of ecosystem functions are among the significant environmental consequences of pesticide use.

These findings emphasise that regulations governing pesticide use should not be based solely on short-term efficacy or target organism control. Instead, a comprehensive approach is required that considers genotoxic risk, bioaccumulation, ecosystem health, and public health. Monitoring pesticide residues, conducting long-term biomonitoring studies, and strengthening risk assessment processes are essential. Additionally, promoting sustainable alternatives such as biological control and integrated pest management is crucial for mitigating genetic and ecological risks.

Future research should employ standardised methodologies to assess different classes of pesticides, exposure routes (occupational, environmental, and dietary), dose–duration relationships, and modulating factors (DNA repair capacity, genetic susceptibility, age, sex, and co-exposures). Such studies will contribute to a more accurate and comprehensive understanding of pesticide genotoxicity.

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Chapter 2

SEASONAL FATTY ACID COMPOSITION OF TOTAL LIPID, TRIACYLGLYCEROL AND PHOSPHOLIPID FRACTIONS IN GONAD AND LIVER OF FEMALE CARASOBARBUS LUTEUS

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INTRODUCTION

Lipids are essential biochemical components in fish, serving critical roles as energy reserves, structural constituents of cellular membranes, and precursors of biologically active molecules. In teleost fish, both the quantity and quality of lipids vary considerably among tissues and are strongly influenced by physiological status, reproductive cycle, environmental conditions, and nutritional availability (Ackman, 1967; Tocher, 2003). Among lipid classes, neutral lipids such as triacylglycerols primarily function as energy storage compounds, whereas polar lipids, particularly phospholipids, are fundamental components of biological membranes and play key roles in cellular integrity and function (Sargent et al., 2002).

Fatty acid composition is especially important in fish reproduction, as developing oocytes require substantial amounts of lipids to support embryogenesis and early larval development. During vitellogenesis, lipids are actively mobilized from storage tissues, notably the liver, and transported to the gonads, resulting in tissue-specific lipid allocation patterns (Wiegand, 1996). Long-chain polyunsaturated fatty acids (PUFA), particularly docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), and arachidonic acid (ARA, 20:4n-6), are known to be essential for membrane fluidity, neural and visual development, and reproductive signaling processes in fish (Sargent et al., 1989; Tocher & Sargent, 2002).

Numerous studies have demonstrated that phospholipid fractions of reproductive tissues are selectively enriched in long-chain PUFA, whereas triacylglycerol fractions are dominated by saturated (SFA) and monounsaturated fatty acids (MUFA), reflecting their respective structural and energetic functions (Bell et al., 1983; Tocher, 1993). This functional differentiation between lipid fractions is considered a conserved metabolic strategy among teleost fish, ensuring the preferential allocation of essential fatty acids to biologically critical tissues during reproduction.

Freshwater cyprinid species have been widely studied with respect to muscle lipid composition; however, detailed information on lipid class distribution and fatty acid profiles of reproductive and metabolic organs remains limited. Existing studies on cyprinids such as *Cyprinus carpio*, *Capoeta* spp., and *Salmo trutta* have reported marked seasonal and tissue-specific variations in

lipid content and fatty acid composition, particularly during the spawning period (Akpınar, 1985; Vlaming et al., 1978; Akpınar et al., 2009). Despite its ecological and regional importance, information on lipid metabolism in *Carasobarbus luteus* is scarce.

Carasobarbus luteus is a freshwater cyprinid species widely distributed in river systems of the Middle East and Anatolia. Understanding its lipid and fatty acid composition, particularly in reproductive tissues, is essential for elucidating species-specific reproductive strategies and metabolic adaptations. Moreover, such data contribute to the broader understanding of lipid utilization patterns in freshwater teleosts and provide a valuable basis for comparative and nutritional studies.

Therefore, the aim of the present study was to determine the total lipid content and fatty acid composition of total lipid, TG, and PL fractions in the gonad and liver tissues of female *Carasobarbus luteus* during the reproductive period. By examining tissue- and lipid class–specific fatty acid distributions, this study seeks to clarify the roles of different lipid fractions in reproductive physiology and to fill an important gap in the existing literature on freshwater cyprinid lipid metabolism.

2. Materials and Methods

Fish samples used in the study were collected from Atatürk Dam Lake using fishing nets in May. Gonad and liver tissues of female fish were excised fresh, their wet weights were determined, and the tissues were stored in chloroform–methanol (2:1, v/v) at −20 °C. Tissues to be analyzed were homogenized in chloroform–methanol (2:1). Non-lipid impurities such as proteins, carbohydrates, and amino acids were removed by washing with 0.88% KCl solution. Total lipids were separated into phospholipid and triacylglycerol fractions by thin-layer chromatography. Fatty acids in the fractions were converted to methyl esters by boiling in acidic methanol. The percentage composition of fatty acids was analyzed by gas chromatography.

RESULTS

In the present study, total lipid content and fatty acid composition of total lipid, TAG and PL fractions were determined in the gonad and liver tissues of female Carasobarbus luteus sampled in May.

Total Lipid Content

The total lipid content expressed on a wet weight basis was 1.60 g/100 g in the gonad and 1.32 g/100 g in the liver (Table 1). Although the gonad exhibited a higher total lipid level than the liver, no statistically significant difference was detected between the tissues ($P > 0.05$) (Figure 1).

Fatty Acid Composition of Total Lipid Fraction

In the total lipid fraction, ∑SFA constituted the predominant group in both tissues, accounting for 41.45% in the gonad and 38.42% in the liver. 16:0 and 18:0 were the major saturated fatty acids in both tissues (Table 2).

Monounsaturated fatty acids (∑MUFA) represented 23.18% of total fatty acids in the gonad and 27.62% in the liver, with 18:1n-9 being the dominant MUFA in both tissues.

Polyunsaturated fatty acids (∑PUFA) were present at 35.24% in the gonad and 33.60% in the liver. DHA and 20:4n-6 were the most abundant PUFA in both tissues. The ω3/ω6 ratio was calculated as 3.09 in the gonad and 2.76 in the liver (Figure 2).

Fatty Acid Composition of Triacylglycerol Fraction

In the TAG fraction, saturated fatty acids were the dominant group, representing 42.05% in the gonad and 48.09% in the liver. 16:0 was the most abundant saturated fatty acid in both tissues.

Monounsaturated fatty acids accounted for 26.13% in the gonad and 32.64% in the liver TAG fraction. Oleic acid (18:1n-9) and palmitoleic acid (16:1n-7) were particularly elevated in the liver.

Polyunsaturated fatty acids comprised 31.60% of total fatty acids in the gonad TAG fraction and 19.18% in the liver. DHA and EPA were the principal n-3 PUFA detected. The ω3/ω6 ratio was 3.11 in the gonad and 2.26 in the liver TAG fraction (Figure 3).

Fatty Acid Composition of Phospholipid Fraction

In the phospholipid fraction, polyunsaturated fatty acids were the predominant group in both tissues, accounting for 39.22% in the gonad and 34.20% in the liver. DHA was the most abundant fatty acid, with values of 21.34% in the gonad and 19.63% in the liver.

Saturated fatty acids constituted 42.99% of gonad phospholipids and 45.62% of liver phospholipids, with stearic acid (18:0) being particularly prominent in the liver.

Total ω3 fatty acids were recorded as 29.05% in the gonad and 26.86% in the liver phospholipid fraction. The ω3/ω6 ratio was 2.85 in the gonad and 3.65 in the liver (Figure 4).

DISCUSSION

The present study provides comprehensive data on total lipid content and fatty acid composition of total lipid, TAG and phospholipid (PL) fractions in the gonad and liver of female *Carasobarbus luteus* during the reproductive period. The observed lipid distribution patterns reflect tissue-specific metabolic functions and are largely consistent with previously reported findings for freshwater teleosts.

Total Lipid Content and Reproductive Allocation

The higher total lipid content detected in the gonad compared with the liver supports the concept that lipid accumulation in reproductive tissues increases during the maturation and spawning periods. In teleost fish, gonadal lipid deposition is closely associated with vitellogenesis, during which lipids are actively transported from storage organs, particularly the liver, to developing oocytes (Wiegand, 1996; Sargent et al., 2002). Similar gonad-dominant lipid accumulation has been reported for *Cyprinus carpio* (Akpınar, 1985) and several cyprinid species inhabiting freshwater ecosystems.

Dominance of SFA and MUFAs in TAG

Saturated fatty acids (SFA), mainly 16:0 and 18:0, constituted a major proportion of total fatty acids in all lipid fractions, particularly in the TAG fraction. This pattern is typical of freshwater fish and reflects the role of TAG as an energy reservoir (Ackman, 1967; Tocher, 2003). The high SFA and MUFA content in liver TAG suggests that this tissue functions as a central lipid storage organ, providing readily oxidizable substrates during periods of increased metabolic demand.

MUFAs, dominated by 18:1n-9, were especially abundant in the liver TAG fraction. Elevated MUFA levels in storage lipids have been associated with enhanced metabolic flexibility and energy mobilization in fish, particularly during reproduction and seasonal temperature changes (Ackman & Takeuchi, 1986; Vlaming et al., 1978).

Enrichment of PUFAs in PLs

In contrast to TAG, phospholipid fractions were characterized by a marked enrichment of PUFAs, particularly long-chain n-3 PUFA such as DHA. The dominance of DHA in gonadal phospholipids is consistent with its essential structural role in biological membranes and its importance for embryonic and larval development (Sargent et al., 1989; Tocher & Sargent, 2002).

The preferential incorporation of DHA into phospholipids rather than neutral lipids has been widely reported in teleost fish and is considered a conserved metabolic strategy to maintain membrane fluidity and functionality, especially in reproductive tissues (Bell & Tocher, 1990; Tocher, 1993).

Role of Arachidonic Acid in Reproductive Physiology

ARA was present at notable levels, particularly in gonadal phospholipids. ARA serves as a precursor for eicosanoids, including prostaglandins, which play critical roles in ovulation, steroidogenesis, and reproductive signaling in fish (Sargent et al., 2002; Wiegand, 1996). The coexistence of relatively high ARA and DHA levels suggests a finely regulated balance between n-6 and n-3 PUFA during the reproductive phase.

Comparable ARA-enriched phospholipid profiles have been reported in the gonads of rainbow trout (*Salmo gairdneri*) and Atlantic cod (*Gadus morhua*), highlighting the conserved nature of this reproductive adaptation among teleosts (Bell et al., 1983; Tocher, 1993).

Tissue-Specific Lipid Fractionation and Selective PUFA Retention

The clear differentiation between TAG and PL fractions observed in the present study underscores their distinct physiological roles. TAG fractions

primarily serve as energy stores, whereas PL fractions are structurally oriented and selectively enriched in essential PUFA. The lower PUFA content in liver TAG compared with gonadal TAG suggests selective retention and/or transport of long-chain PUFA to reproductive tissues, a phenomenon previously documented in cyprinids and other freshwater species (Ackman et al., 2002; Akpınar et al., 2009).

ω3/ω6 Ratio and Nutritional Perspective

The relatively high ω3/ω6 ratios observed in gonadal and phospholipid fractions indicate a dominance of n-3 PUFA, a characteristic feature of freshwater fish lipids (Arts et al., 2001). From a physiological standpoint, elevated ω3/ω6 ratios are associated with optimized membrane function and reproductive success, while from a nutritional perspective, they enhance the dietary value of fish for human consumption.

Overall Implications

In summary, the lipid and fatty acid profiles of female *C. luteus* demonstrate a coordinated pattern of lipid storage and utilization that supports reproductive processes. The enrichment of long-chain PUFA in gonadal phospholipids, combined with energy-dense TAG reserves in the liver, reflects a metabolic strategy that has been widely documented in teleost fish. These findings contribute valuable baseline data for *C. luteus* and provide a framework for future studies examining seasonal variation, dietary influences, and reproductive energetics in freshwater cyprinids.

CONCLUSION

The present study provides detailed insight into the tissue-specific lipid distribution and fatty acid composition of total lipid, TG, and PL fractions in the gonad and liver of female *C. luteus* during the reproductive period. The results demonstrate that lipid allocation differs markedly between tissues and lipid classes, reflecting their distinct physiological functions.

The gonad was characterized by a higher proportion of PUFAs, particularly long-chain n-3 fatty acids such as docosahexaenoic acid, especially within the phospholipid fraction. This enrichment highlights the critical structural and

functional role of polyunsaturated fatty acids in reproductive tissues, supporting membrane integrity and processes associated with oocyte development. In contrast, the liver exhibited higher levels of saturated and monounsaturated fatty acids in the triacylglycerol fraction, consistent with its role as a primary site of energy storage and metabolic regulation.

The clear differentiation between triacylglycerol and phospholipid fractions underscores a conserved metabolic strategy in teleost fish, whereby energy-dense lipids are segregated from structurally essential membrane lipids. The relatively high ω3/ω6 ratios observed across lipid fractions further indicate the dominance of n-3 polyunsaturated fatty acids and emphasize their importance in reproductive physiology.

Overall, these findings contribute novel baseline data on lipid metabolism in *C. luteus* and enhance current understanding of lipid utilization strategies in freshwater cyprinids. The results provide a foundation for future studies investigating seasonal variation, dietary influences, and environmental factors affecting lipid dynamics and reproductive performance in this species.

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Figure 1

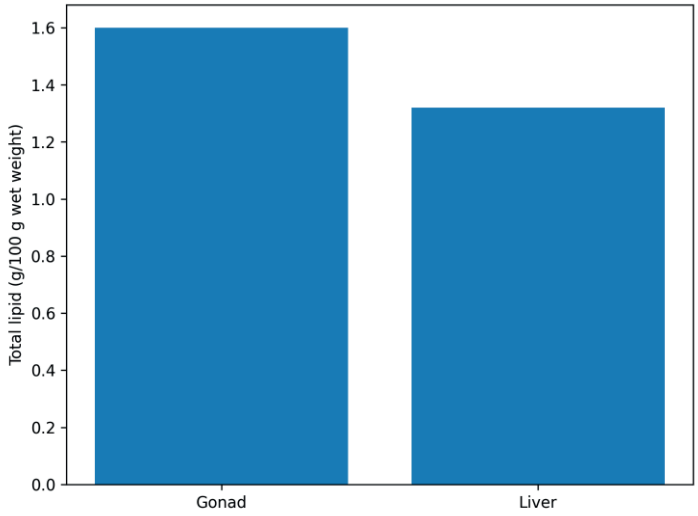

Figure 1. Total lipid content (g/100 g wet weight) in gonad and liver tissues of female *C. luteus*.

Figure 2

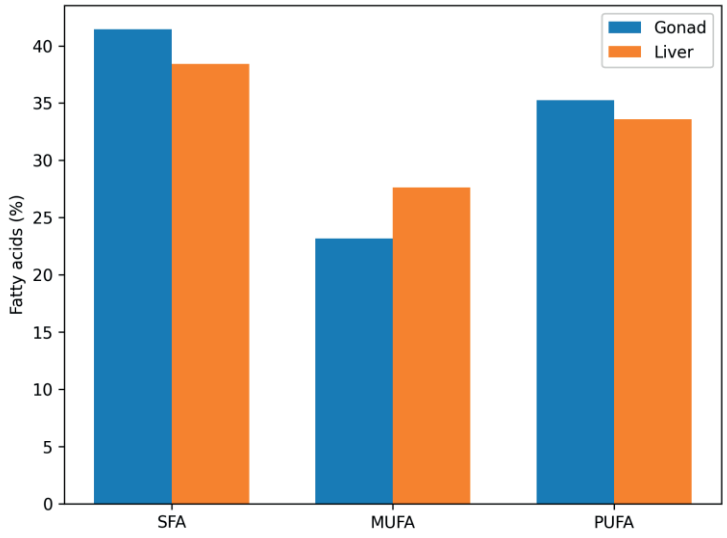

Figure 2. Distribution of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids in the total lipid fraction of gonad and liver tissues of female *C. luteus*.

Figure 3

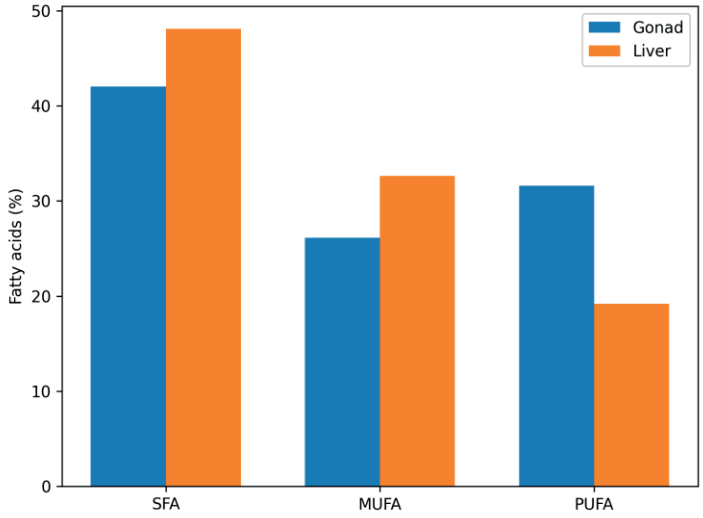

Figure 3. Fatty acid class composition of the triacylglycerol fraction in gonad and liver tissues of female *C. luteus*.

Figure 4

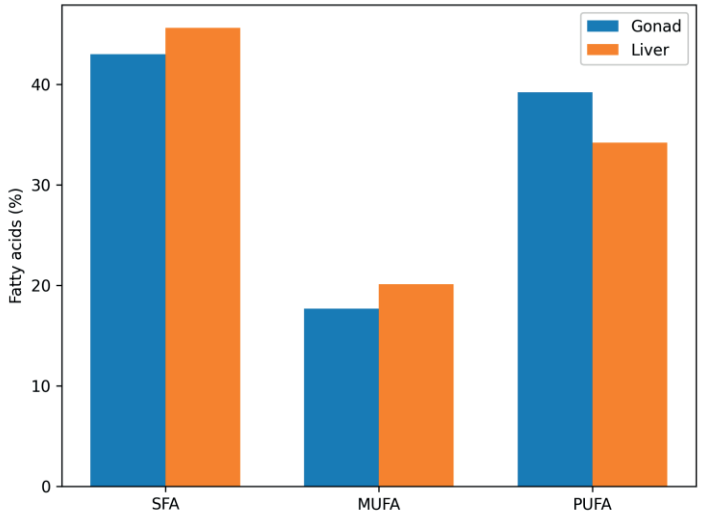

Figure 4. Fatty acid class composition of the phospholipid fraction in gonad and liver tissues of female *C. luteus*.

Table 2- Fatty acid composition in total lipid, triacylglycerol and phospholipid fraction of gonad and liver
from female *C. luteus* (% of total FA)*

	Total		triacylglycerol		phospholipid	
Fatty acid	gonad	liver	gonad	liver	gonad	liver
10:0*	-	-	0.03±0.01a*	-	-	-
12:0	0.01±0.01a	-	0.04±0.01b	-	-	-
13:0	0.04±0.02a	0.17±0.03b	0.09±0.02c	-	-	-
14:0	2.32±0.05a	2.55±0.06a	2.60±0.01a	5.10±0.04b	0.82±0.01c	0.80±0.02c
15:0	0.45±0.04a	0.47±0.05a	0.49±0.04a	0.75±0.01b	0.36±0.02c	0.29±0.03c
16:0	27.06±1.20 a	24.85±1.22 a	27.77±1.09 a	33.81±1.97b	25.63±1.56 a	24.51±1.45 a
17:0	0.37±0.03a	0.01±0.01b	0.33±0.02a	0.44±0.04c	0.13±0.03d	0.21±0.01e
18:0	11.20±1.66 a	10.37±1.98 a	10.70±1.06 a	7.99±0.99b	16.05±1.56 c	19.81±1.50 d
ΣS.F.A** *	**41.45**±2.34 a	**38.42**±2.88 b	**42.05**±2.09 a	**48.09**±2.00c	**42.99**±2.34 a	**45.62**±2.87 c
16:1n-7	4.22±0.99a	6.90±0.76b	6.80±0.66b	10.14±1.24c	2.65±0.98d	2.96±0.78d
18:1n-9	17.80±1.25 a	19.27±1.47 a	18.15±1.76 a	21.26±1.09b	14.09±1.56 c	16.17±1.33 a
20:1n-9	1.16±0.99a	1.45±0.45b	1.18±0.56a	1.24±0.05a	0.95±0.03a	0.96±0.08a
ΣM.U.F.A	**23.18**±1.22 a	**27.62**±1.55 b	**26.13**±1.89 b	**32.64**±1.67c	**17.69**±1.20 d	**20.09**±1.05 a
18:2n-6	1.59±0.06a	2.45±0.07b	1.31±0.01a	1.87±0.56a	0.86±0.05c	0.95±0.08c
18:3n-3	0.62±0.07a	1.83±0.04b	0.63±0.03a	1.04±0.09b	0.26±0.03c	0.39±0.01c
20:2n-6	0.34±0.02a	0.38±0.03a	0.34±0.01a	0.53±0.04b	0.35±0.04a	0.45±0.01b
20:3n-6	0.41±0.04a	1.00±0.01b	0.45±0.06a	0.30±0.01c	0.41±0.03a	0.34±0.02c
20:4n-6	6.26±0.98a	5.10±0.80a	5.58±0.76a	3.18±0.67b	8.55±0.45c	5.60±0.44a
20:5n-3	4.84±0.45a	4.13±0.23a	4.56±0.40a	3.16±0.33b	4.92±0.76a	3.93±0.45a
22:5n-3	2.94±0.67a	2.75±0.55a	2.96±0.40a	1.50±0.34b	2.53±0.33a	2.91±0.59a
22:6n-3	18.24±1.87 a	15.96±1.67 b	15.77±1.78 b	7.60±1.00c	21.34±1.56 d	19.63±1.67 a
ΣP.U.F.A	**35.24**±2.08 a	**33.60**±2.45 a	**31.60**±2.67 a	**19.18**±2.07b	**39.22**±2.35 c	**34.20**±1.09 a
n-3	26.64	24.67	23.92	13.3	29.05	26.86
n-6	8.60	8.93	7.68	5.88	10.17	7.34
n-3/n-6	3.09	2.76	3.11	2.26	2.85	3.65

* Means are the averages of 3 replicates

** Values reported are means ±standard error; means followed by different letters in same line are significantly different (p<0.05) by Tukey's test. *** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

Table 1: Total lipid of gonad and liver tissue of female *C. luteus*

Tissue	Total lipid (g)
Total lipids of gonad (%)	1.60
Total lipids of liver (%)	1.32

Chapter 3

LICHEN SECONDARY METABOLITES AND BIOLOGICAL ACTIVITIES

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General Characteristics of Lichens

Lichens are organisms formed through a mutualistic symbiosis between a fungal mycobiont and a photobiont of algal or cyanobacterial origin, exhibiting a remarkable capacity to adapt to extreme environmental conditions (Elix & Stocker-Wörgötter, 2008). Owing to this symbiotic structure, lichens are able to survive on nutrient-poor substrates such as rock surfaces, tree bark, and soil, and are considered among the pioneer colonizers in many ecosystems (Elix & Stocker-Wörgötter, 2008). It is widely accepted that secondary metabolites synthesized by lichens play a decisive role in this ecological success (Ingólfsdóttir, 2002).

Lichen secondary metabolites mainly consist of compounds that are largely unique to these organisms, including depsides, depsidones, dibenzofuran derivatives, and various phenolic substances (Ingólfsdóttir, 2002). These compounds primarily function in photoprotection against UV radiation, chemical defense against herbivores and microorganisms, and limitation of the harmful effects of oxidative stress (Solhaug & Gauslaa, 1996). In particular, metabolites such as usnic acid, atranorin, and fumarprotocetraric acid are among the most extensively investigated molecules in lichen biochemistry due to their chemotaxonomic and pharmacological significance (Cansaran et al., 2006; Ingólfsdóttir, 2002).

Recent studies have demonstrated that lichens possess not only ecological importance but also considerable potential for biomedical and biotechnological applications (Kosanić et al., 2013). Numerous in vitro and cell-based investigations have shown that lichen extracts and isolated pure metabolites exhibit antioxidant, antimicrobial, DNA-protective, and anticancer properties (Mitrović et al., 2011; Sepahvand et al., 2021). These findings position lichens as strategic natural sources for the discovery and development of novel bioactive agents (Kello et al., 2023).

Within the scope of this study, the selection of species belonging to the genera *Usnea*, *Evernia*, *Parmelia*, *Cladonia*, and *Ramalina* is mainly based on their

documented diversity of secondary metabolites and broad spectra of biological activities (Ingólfsdóttir, 2002; Elix & Stocker-Wörgötter, 2008). *Usnea* species are distinguished by their high usnic acid content and pronounced antimicrobial activity, whereas *Evernia* species draw attention due to their richness in phenolic compounds and strong antioxidant capacity (Kosanić et al., 2013; Shcherbakova et al., 2021). *Parmelia* species, characterized predominantly by depsidone-based metabolite profiles and selective cytotoxic effects, are regarded as an important group in pharmacological research (Cansaran et al., 2006; Aslan Engin et al., 2023). Additionally, *Cladonia* species stand out with their chemical diversity as well as antimicrobial, antioxidant, and genoprotective effects, and their holobiont structure offers substantial potential for the discovery of novel natural products (Mitrović et al., 2011; Shishido et al., 2021). *Ramalina* species, rich particularly in phenolic and depside-derived metabolites, are also notable in lichen biochemistry literature for their significant antioxidant activity and suitability for biotechnological applications (Kosanić et al., 2013; Kello et al., 2023).

Evaluating these five genera together enables a comparative assessment of different metabolite classes and their biological effects, thereby providing a more comprehensive understanding of the pharmaceutical, cosmetic, and biotechnological potential of lichen-derived bioactive compounds (Mitrović et al., 2011; Kello et al., 2023). Therefore, this study aims to make a substantial contribution to the lichen biochemistry literature and to establish a strong scientific basis for future experimental and applied research (Sepahvand et al., 2021).

Usnea Species

1. Phytochemical Content and Metabolite Profile

Lichens of the genus *Usnea* are among the most extensively investigated groups in the literature with respect to the diversity of their secondary metabolites. The phytochemical profile of this genus predominantly consists of

dibenzofuran derivatives, depsides, depsidones, and various phenolic compounds (Ingólfsdóttir, 2002). In particular, usnic acid is considered the characteristic metabolite of *Usnea* species and is regarded as a key compound in chemotaxonomic evaluations (Cansaran et al., 2006).

Usnic acid [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-dibenzofuran-1,3-dione] is a molecule unique to lichens and plays a protective role against environmental stress factors (Ingólfsdóttir, 2002). Additionally, other secondary metabolites such as evernic acid, barbatic acid, and diffractic acid have been reported in varying proportions among different *Usnea* species (Elix & Stocker-Wörgötter, 2008).

Recent analytical studies employing advanced techniques such as UHPLC, HPTLC–MS, and UPLC–QToF-MS have demonstrated that previously unreported metabolites may also be identified in *Usnea* species (Salgado et al., 2017; Tatapudi et al., 2025). The amount of usnic acid has been shown to vary significantly not only among species but also among samples of the same species collected from different geographical regions, and this variation has been associated with environmental factors such as substrate characteristics, light intensity, and ecological conditions (Cansaran et al., 2006; Kocakaya et al., 2024). Indeed, studies on Anatolian *Usnea* species have revealed that usnic acid content may fluctuate over a wide range depending on dry weight, and this variation may be critical in determining biological activity (Cansaran et al., 2006). It has further been emphasized that metabolite levels may differ substantially both among species and among individuals of the same species growing in different habitats, and that this variability is associated with environmental parameters such as light intensity, substrate type, and geographical location (Ji & Khan, 2005; Maulidiyah et al., 2023).

2. Antioxidant Activity and Effects Related to Oxidative Stress

The antioxidant capacity of *Usnea* species is directly associated with their phenolic constituents, particularly usnic acid. Considering the role of oxidative stress in cellular damage, aging, and the pathogenesis of many chronic diseases, the free radical scavenging potential of *Usnea* extracts has become an

important research focus (Salgado et al., 2017). In vitro antioxidant assays (DPPH, ABTS, and FRAP) have demonstrated that methanolic extracts obtained from *Usnea* species exhibit significant radical scavenging activity (Maulidiyah et al., 2023). This effect is thought to result from the electron-donating properties of usnic acid due to its phenolic structure (Ingólfsdóttir, 2002).

Moreover, DNA-protective studies have shown that *Usnea* extracts reduce UV- and H_2O_2-induced oxidative DNA damage. Investigations using the pBR322 plasmid DNA model indicate that *Usnea* species may support antioxidant defense not only at the chemical level but also in biological systems (Sepahvand et al., 2021).

3. Antimicrobial and Antifungal Activity

Consistent with their long history of traditional use, *Usnea* species exhibit strong antimicrobial properties. Numerous studies have reported pronounced inhibitory activity particularly against Gram-positive bacteria (Kınalıoğlu et al., 2019). Usnic acid is considered the primary compound responsible for this effect. Extracts obtained from species such as *Usnea longissima* and *U. barbata* have been reported to form significant inhibition zones against *Staphylococcus aureus* and *Bacillus subtilis* (Cansaran et al., 2006; Kınalıoğlu et al., 2019). The antimicrobial mechanism is thought to involve disruption of bacterial cell membrane integrity and suppression of energy metabolism.

The antifungal and antimycotic potential of *Usnea* extracts has also been emphasized as clinically relevant, offering promising prospects for pharmacological research (Guo et al., 2008). In terms of antifungal activity, *Usnea* species have been reported to exhibit moderate effects against *Candida albicans*, whereas their activity against filamentous fungi appears to be more limited (Kınalıoğlu et al., 2019).

4. Anticancer and Cytotoxic Potential

Recent cell culture–based studies have highlighted the anticancer potential of *Usnea* species. Both *Usnea* extracts and pure usnic acid have demonstrated

inhibitory effects on the proliferation of various cancer cell lines. Studies conducted on breast, gastric, and colon cancer cells have revealed that this effect is dose-dependent (Aslan Engin et al., 2023). Mechanistic investigations suggest that this cytotoxicity may involve cell-cycle arrest, induction of apoptosis via mitochondrial pathways, and alterations in reactive oxygen species homeostasis (Guo et al., 2008).

An important aspect noted in several studies is that usnic acid may exhibit higher cytotoxicity in cancer cells compared to normal cells (Aslan Engin et al., 2023). Nevertheless, the potential hepatotoxic effects of high doses of usnic acid have also been reported. Therefore, when considering *Usnea*-derived compounds for anticancer applications, dosage, formulation, and carrier systems must be carefully evaluated (Guo et al., 2008).

5. Pharmaceutical, Cosmetic, and Biotechnological Importance

Due to their phenolic metabolites and usnic acid content, *Usnea* species are regarded as strategic natural resources for pharmaceutical, cosmetic, and biotechnological applications. Their antioxidant and antimicrobial properties support potential use as natural preservative agents, bioactive coating materials, and pharmaceutical additives (Ingólfsdóttir, 2002; Mitrović et al., 2011). Additionally, reliable quantitative determination of usnic acid is of great importance for quality control processes and standardization of commercial products (Ji & Khan, 2005; Tatapudi et al., 2025).

In cosmetic applications, *Usnea* extracts are employed particularly in natural formulations to prevent microbial contamination and reduce oxidative stress. Furthermore, in biotechnological research, *Usnea* species are considered promising raw materials for natural antimicrobial coatings and biodegradable packaging systems (Elix & Stocker-Wörgötter, 2008).

Overall, these findings clearly demonstrate that *Usnea* species constitute a significant research subject not only from ecological and biochemical perspectives but also in relation to therapeutic and industrial applications.

Evernia Species

1. Phytochemical Content and Metabolite Profile

Evernia prunastri is an important lichen species of pharmacological interest due to its rich secondary metabolite content and broad spectrum of biological activities. LC-HRMS/MS and HPLC-based chemical analyses have revealed that this species is particularly rich in depsides (evernic acid, atranorin), physodic acid, and various phenolic compounds (Stojković et al., 2025; Kosanić et al., 2013). This chemical diversity forms the basis of the antioxidant, antimicrobial, and cytotoxic activities of *E. prunastri*.

Metabolite production in *Evernia* species is highly sensitive to environmental conditions. In particular, light intensity and humidity directly influence secondary metabolite synthesis. Compounds such as atranorin and chloroatranorin have been reported to play a photoprotective role against increased UV exposure under high-light conditions. This is considered an important adaptation mechanism explaining the widespread occurrence of *Evernia* species in epiphytic habitats (Solhaug & Gauslaa, 2004).

Analytical studies using advanced techniques such as HPLC and LC-MS/MS have enabled detailed characterization of the metabolite profiles of *Evernia* species. Studies conducted especially on *Evernia prunastri* have demonstrated that concentrations of usnic acid and atranorin may vary significantly depending on species characteristics and habitat conditions (Cansaran et al., 2006). This variability forms the basis of the differences observed in the biological activities of *Evernia* species.

2. Antioxidant Activity and Effects Related to Oxidative Stress

Evernia species possess a marked antioxidant potential owing to their high phenolic content. Considering the role of oxidative stress in cellular damage, inflammation, and aging processes, the free radical scavenging properties of *Evernia* extracts hold considerable biological significance (Salgado et al., 2017).

In vitro antioxidant assays have demonstrated that *Evernia prunastri* extracts effectively neutralize DPPH and ABTS radicals, an effect thought to be associated with the electron-donating capacities of phenolic compounds (Ingólfsdóttir, 2002). FRAP analyses have also reported notable reducing capacity in *Evernia* species (Salgado et al., 2017).

In studies evaluating DNA-protective effects, *Evernia* extracts have been shown to significantly reduce UV- and hydrogen peroxide–induced oxidative DNA damage. Investigations using plasmid DNA models demonstrate that the antioxidant effects of *Evernia* species are also relevant in biological systems (Sepahvand et al., 2021).

3. Antimicrobial and Antifungal Activity

The antimicrobial properties of *Evernia* species have been particularly reported against Gram-positive bacteria. Disk diffusion and MIC studies have shown that extracts of *Evernia prunastri* and *E. mesomorpha* exert significant inhibitory effects against pathogens such as *Staphylococcus aureus* and *Bacillus subtilis* (Kınalıoğlu et al., 2019).

The antimicrobial mechanism is associated with disruption of cell membrane integrity and suppression of bacterial enzymatic systems. Compounds such as atranorin and usnic acid are thought to increase membrane permeability. The relatively limited effectiveness against Gram-negative bacteria is attributed to the structural characteristics of their outer membrane (Ingólfsdóttir, 2002).

Studies conducted on hexane, dichloromethane, and acetonitrile extracts have demonstrated that *E. prunastri* exhibits particularly strong antimicrobial activity against Gram-positive bacteria. A pronounced inhibitory effect has been observed against *Staphylococcus aureus*, and the acetonitrile extract has also been reported to be effective against *Candida albicans*. NMR analyses indicated that evernic acid is the primary determinant of this biological activity, demonstrating that the effect is not limited solely to usnic acid (Shcherbakova et al., 2021).

4. Anticancer and Cytotoxic Potential

The anticancer potential of *Evernia* species has gained increasing attention in recent years owing to cell culture-based studies. Extracts of *Evernia* and their constituents, such as usnic acid and atranorin, have been reported to suppress cell proliferation in various cancer cell lines (Aslan Engin et al., 2023).

In particular, *Evernia prunastri* extracts have been shown to exert significant cytotoxic effects on MCF-7 breast cancer and HeLa cervical cancer cells, while exhibiting relatively lower toxicity toward normal cells (Aslan Engin et al., 2023). This selective activity allows *Evernia* species to be considered among potential complementary anticancer agents.

Mechanistic evaluations suggest that cytotoxic effects may involve cell-cycle arrest, induction of apoptosis, and alterations in intracellular reactive oxygen species balance (Guo et al., 2008). However, further advanced pharmacological and toxicity studies are required before these findings can be translated into clinical applications.

5. Pharmaceutical, Cosmetic, and Biotechnological Importance

Due to their rich metabolite composition and broad range of biological activities, *Evernia* species are considered important natural resources for pharmaceutical and cosmetic applications. Their antimicrobial and antioxidant properties make these lichens valuable as natural preservative agents and bioactive components (Ingólfsdóttir, 2002).

In the cosmetic industry, *Evernia* extracts are particularly used in natural-based formulations to prevent microbial contamination and reduce oxidative stress. Atranorin and phenolic compounds have been reported to exert protective effects on the skin (Guo et al., 2008).

From a biotechnological perspective, *Evernia* species are considered potential raw materials for natural antimicrobial coatings, bioactive packaging systems, and development of plant-derived active compounds. In this regard, the genus

Evernia holds not only ecological importance but also strategic value for industrial and biomedical applications.

Parmelia Species

1. Phytochemical Content and Metabolite Profile

Lichens of the genus *Parmelia* are among the best-characterized taxa in lichen biochemistry with respect to secondary metabolite diversity (Ingólfsdóttir, 2002). Their phytochemical profile mainly consists of depsides, depsidones, and various phenolic compounds, which not only provide protection against environmental stressors but also constitute the biochemical basis of their biological activities (Elix & Stocker-Wörgötter, 2008).

Among the most commonly reported secondary metabolites in *Parmelia* species are atranorin, salazinic acid, protocetraric acid, and fumarprotocetraric acid (Cansaran et al., 2006). Atranorin is generally localized in the cortical layer, whereas salazinic and protocetraric acids are predominantly accumulated in the medulla (Elix & Stocker-Wörgötter, 2008). The distribution of these compounds serves as an important chemotaxonomic criterion for distinguishing *Parmelia* species (Ingólfsdóttir, 2002).

The quantity and composition of metabolite production are determined not only by species characteristics but are also strongly influenced by environmental factors such as light intensity, substrate type, altitude, and geographical location (Cansaran et al., 2006). HPLC- and LC-MS/MS–based analyses have demonstrated significant habitat-dependent variability in the levels of atranorin and depsidone derivatives (Ji & Khan, 2005). This variability forms the basis for the differences observed in the biological activities of *Parmelia* species (Maulidiyah et al., 2023).

2. Antioxidant Activity and Effects Related to Oxidative Stress

The antioxidant potential of *Parmelia* species is closely associated with their high phenolic content, particularly depside- and depsidone-type compounds (Ingólfsdóttir, 2002). Owing to the electron-donating capacity of phenolic

hydroxyl groups, these compounds effectively neutralize free radicals, thereby contributing to the reduction of oxidative stress–induced cellular damage (Salgado et al., 2017).

In vitro antioxidant assays have reported that methanolic and ethanolic extracts of species such as *Parmelia sulcata* and *Parmelia saxatilis* exhibit significant DPPH and ABTS radical scavenging activities (Maulidiyah et al., 2023). FRAP analyses have likewise revealed notable reducing capacities in *Parmelia* extracts (Salgado et al., 2017). This antioxidant effect has been particularly correlated with the presence of atranorin and fumarprotocetraric acid (Ingólfsdóttir, 2002).

Studies evaluating DNA-protective properties have demonstrated that *Parmelia* extracts reduce UV- and hydrogen peroxide–induced oxidative DNA damage (Sepahvand et al., 2021). Findings obtained using plasmid DNA models indicate that *Parmelia* species may help support oxidative balance not only at the chemical level but also within biological systems (Sepahvand et al., 2021).

3. Antimicrobial and Antifungal Activity

Parmelia species are among the earliest lichen genera reported to possess antimicrobial properties, with particularly strong inhibitory effects against Gram-positive bacteria (Kınalıoğlu et al., 2019). Extracts of *Parmelia sulcata* and *Parmelia perlata* have been shown to produce significant inhibition zones against *Staphylococcus aureus* and *Bacillus subtilis* (Cansaran et al., 2006).

Their antimicrobial activity is primarily attributed to depsidone and phenolic compounds that disrupt bacterial cell membrane integrity and suppress energy metabolism (Ingólfsdóttir, 2002). MIC studies have indicated that in some cases, *Parmelia* extracts exhibit effects comparable to standard antibiotics (Kınalıoğlu et al., 2019).

In terms of antifungal activity, *Parmelia* species have demonstrated moderate inhibitory effects against *Candida albicans*, whereas their activity against filamentous fungi appears to be relatively limited (Kınalıoğlu et al., 2019).

These findings suggest that *Parmelia* extracts may hold greater promise particularly in antibacterial applications (Ingólfsdóttir, 2002).

4. Anticancer and Cytotoxic Potential

In recent years, cell culture–based studies have increasingly emphasized the anticancer potential of *Parmelia* species (Aslan Engin et al., 2023). Extracts of *Parmelia sulcata* and *Parmelia saxatilis* have been reported to inhibit cell proliferation and exert dose-dependent cytotoxic effects in various cancer cell lines (Aslan Engin et al., 2023).

Mechanistic evaluations suggest that cytotoxicity may involve cell-cycle arrest, disruption of mitochondrial membrane potential, and induction of apoptosis (Guo et al., 2008). Some studies also indicate that *Parmelia*-derived compounds exert more selective effects on cancer cells compared with normal cells, which is considered promising in terms of complementary therapeutic approaches (Aslan Engin et al., 2023).

However, it has also been emphasized that depsidone and phenolic compounds may exhibit cytotoxicity at high concentrations, underscoring the need to determine safe usage limits (Guo et al., 2008).

5. Pharmaceutical, Cosmetic, and Biotechnological Importance

Due to their diverse biological activities, *Parmelia* species are regarded as potential natural resources for pharmaceutical and cosmetic applications (Ingólfsdóttir, 2002). Their antimicrobial and antioxidant properties make these lichens promising candidates as natural preservatives and bioactive components (Elix & Stocker-Wörgötter, 2008).

In the cosmetic industry, *Parmelia* extracts are suggested for use in natural formulations aimed at reducing oxidative stress and preventing microbial contamination (Guo et al., 2008). Atranorin and phenolic constituents have been reported to exert protective and stabilizing effects on the skin (Ingólfsdóttir, 2002).

From a biotechnological perspective, *Parmelia* species are considered valuable raw materials for the development of natural antimicrobial coatings, bioactive packaging systems, and sustainable biomaterials (Elix & Stocker-Wörgötter, 2008).

Ramalina Species

1. Phytochemical Content and Metabolite Profile

Lichens of the genus *Ramalina*, belonging to the family Parmeliaceae, are foliose lichens that attract attention due to their remarkable diversity of secondary metabolites (González-Burgos et al., 2019). The phytochemical profile of this genus predominantly consists of depsides, depsidones, dibenzofuran derivatives, and various phenolic compounds (Sharma et al., 2013). Owing to their unique metabolites associated with adaptation to environmental stress conditions, *Ramalina* species have received considerable interest both ecologically and pharmacologically (González-Burgos et al., 2019).

One of the most characteristic metabolites of the genus *Ramalina* is ramalin, which was particularly isolated from the Antarctic species *Ramalina terebrata* (Paudel et al., 2010). Ramalin has been highlighted in the literature due to its strong antioxidant, antibacterial, and neuroprotective activities (Kim et al., 2021). In addition, secondary metabolites such as usnic acid, atranorin, and various usimine derivatives are also commonly reported in *Ramalina* species (Paudel et al., 2010).

LC-ESI-MS/MS analyses conducted on *Ramalina sinensis* revealed the presence of 33 different compounds in acetone and methanol extracts, demonstrating that *Ramalina* species possess a rich and complex metabolic profile (Koopaie et al., 2023). It has also been reported that metabolite composition varies not only among species but also depending on solvent type and extraction conditions (Koopaie et al., 2023).

2. Antioxidant Activity and Effects Related to Oxidative Stress

The antioxidant capacity of *Ramalina* species is directly associated with their phenolic constituents, particularly with unique metabolites such as ramalin (Kim et al., 2021). Strengthening antioxidant defense mechanisms plays a critical role in preventing oxidative stress–related pathological conditions, including neurodegenerative diseases and cancer (Kim et al., 2021).

Studies conducted on *Ramalina roesleri* have reported that the DPPH radical scavenging activity of extracts prepared with different solvents ranges between 29.42% and 87.90% (Sisodia et al., 2013). Especially phenolic compounds such as sekikaic acid and homosekikaic acid have been shown to exhibit strong antioxidant capacity (Sisodia et al., 2013). These findings clearly indicate that the antioxidant activity observed in *Ramalina* species is closely associated with specific metabolites (Sisodia et al., 2013).

Research focusing on ramalin has demonstrated that this compound effectively neutralizes reactive oxygen species and limits oxidative cellular damage (Kim et al., 2021). These properties enable *Ramalina*-derived metabolites to be considered as promising antioxidant-based protective agents (Kim et al., 2021).

3. Antimicrobial and Antibacterial Activity

Ramalina species exhibit pronounced antibacterial activity, particularly against Gram-positive bacteria. Extracts of *Ramalina roesleri* have shown strong inhibitory effects against *Staphylococcus aureus* and *Streptococcus mutans*, and this activity has been associated with metabolites such as atranorin, usnic acid, protolichesterinic acid, and sekikaic acid (Sisodia et al., 2013).

Ramalin, usnic acid, and usimine derivatives isolated from the Antarctic species *Ramalina terebrata* have been reported to exert potent antibacterial activity with low MIC values against *Bacillus subtilis* (Paudel et al., 2010). These metabolites are thought to act by disrupting bacterial cell wall integrity and suppressing metabolic processes.

Overall, these findings indicate that *Ramalina* species possess significant potential as natural antimicrobial agents, which is particularly valuable in an era of increasing antibiotic resistance.

4. Anticancer and Cytotoxic Potential

Ramalin derived from *Ramalina* species has drawn considerable attention not only as an antioxidant compound but also as a potent anticancer metabolite. Ramalin obtained from *Ramalina terebrata* has been shown to inhibit proliferation, induce apoptosis, and cause G2/M phase arrest with cytostatic effects in HCT116 human colorectal cancer cells. This effect has been associated with increased expression of TP53 and CDKN1A, along with the suppression of CDK1 and CCNB1 (Suh et al., 2017).

Moreover, ramalin has been identified as a multi-target agent in neurodegenerative processes such as Alzheimer's disease. Through its antioxidant, anti-inflammatory, and BACE-1 inhibitory properties, ramalin has been shown to simultaneously modulate amyloid-β production, oxidative stress, and neuroinflammation. However, its low stability in aqueous environments and dose-dependent cytotoxicity limit its direct clinical applicability. Therefore, ramalin derivatives such as RA-25Me and RA-34Me, which exhibit improved pharmacokinetic properties, have been proposed as more promising therapeutic candidates (Kim et al., 2021).

5. Pharmaceutical and Biotechnological Importance

Recent findings indicate that *Ramalina* species hold substantial potential for pharmaceutical and biotechnological research due to their antioxidant, antimicrobial, anticancer, and neuroprotective properties. Particularly, ramalin has emerged as a strong candidate in dermatology and immunology owing to its ability to suppress NF-κB and MAPK signaling pathways, reduce cytokine production in keratinocytes and mast cells, and alleviate atopic dermatitis–like lesions (Park et al., 2016).

The bioprotective potential of *Ramalina farinacea* against environmental toxins (Kalefetoğlu Macar et al., 2025), the phenolic metabolite–mediated

antimicrobial and antioxidant capacity of *Ramalina roesleri* (Sisodia et al., 2013), and the anticancer and neuroprotective activities of ramalin derived from *R. terebrata* (Suh et al., 2017; Kim et al., 2021) collectively demonstrate that *Ramalina* species represent one of the most promising lichen groups for developing next-generation natural therapeutic agents in modern medicine.

Cladonia Species

1. Phytochemical Composition and Metabolite Profile

Lichens belonging to the genus *Cladonia* are considered among the most remarkable lichen groups in the literature in terms of chemical diversity and secondary metabolite biosynthesis (Shishido et al., 2021). The phytochemical profile of this genus mainly consists of depsides, depsidones, dibenzofuran derivatives, and various phenolic compounds, and it has been reported that the metabolite composition differs significantly among species (Sepúlveda et al., 2022). Recent metagenomic and metabolomic studies have revealed that *Cladonia* species exhibit a complex holobiont structure, in which metabolite production is not solely dependent on the fungal component but also involves algal and bacterial partners (Heuberger et al., 2025). Reference metagenomic analyses particularly performed on *Cladonia rangiformis* demonstrated the presence of numerous secondary metabolite biosynthetic gene clusters encoded by fungi, algae, and bacteria (Heuberger et al., 2025). Advanced chromatographic and mass spectrometric analyses have shown that biologically active compounds such as fumarprotocetraric acid derivatives, atranorin, orsellinic acid, lecanoric acid, gyroforic acid, and lobaric acid are commonly found in *Cladonia* species (Sepúlveda et al., 2022; Dimitrijević et al., 2023). The fact that some of these metabolites have been identified for the first time in the genus *Cladonia* suggests that these lichens represent an important source for the discovery of new natural products (Sepúlveda et al., 2022).

2. Antioxidant Activity and Effects Related to Oxidative Stress

The antioxidant potential of *Cladonia* species is directly associated with their phenolic constituents and depside/depsidone derivatives (Us et al., 2023). Considering that oxidative stress plays a critical role in cellular damage, impairment of DNA integrity, and the development of chronic diseases, the antioxidant properties of *Cladonia* extracts have been extensively investigated (Ranković et al., 2011). Evaluations conducted using multiple in vitro assays such as DPPH, ABTS, CUPRAC, and FRAP have reported significant free radical scavenging activity in extracts of *Cladonia rangiformis*, *C. chlorophaea*, and *C. gracilis* (Torres-Benítez et al., 2022; Dimitrijević et al., 2023). This effect is suggested to arise from the electron-donating capacity of phenolic hydroxyl groups (Ranković et al., 2011). In addition, studies assessing DNA-protective effects have demonstrated that *Cladonia* extracts significantly reduce UV- and H_2O_2-induced oxidative DNA damage (Ceylan et al., 2022). Conducted using human lymphocytes and pBR322 plasmid DNA models, these investigations indicate that *Cladonia* species may support antioxidant defense not only at the chemical level but also in biological systems (Ceylan et al., 2022; Dimitrijević et al., 2023).

3. Antimicrobial and Antifungal Activity

Cladonia species are regarded as natural antibacterial agents due to their broad-spectrum antimicrobial activity (Ranković et al., 2011). Studies carried out on methanolic extracts of various *Cladonia* species collected from Türkiye have reported pronounced inhibitory effects against both Gram-positive and Gram-negative bacteria (Ceylan et al., 2022). Recent investigations conducted with *Cladonia foliacea* extracts demonstrated strong antimicrobial effects against both standard pathogens and methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates (Güneş & Akgül, 2025). Minimum inhibitory concentration analyses revealed that *Bacillus cereus* exhibited particularly high susceptibility to *Cladonia* extracts (Güneş & Akgül, 2025). With respect to antifungal activity, *Cladonia* species generally display moderate inhibitory effects against yeasts and filamentous fungi; however, antibacterial activity

appears to be more prominent compared to antifungal effects (Ceylan et al., 2022; Ranković et al., 2011). This finding suggests that *Cladonia* extracts may be particularly advantageous for antibacterial applications (Us et al., 2023).

4. Anticancer and Cytotoxic Potential

The cytotoxic and anticancer potential of *Cladonia* species has been examined in greater detail in recent years through cell culture–based studies (Ranković et al., 2011). Extracts of *Cladonia furcata* have been reported to show marked cytotoxic effects with low IC_{50} values against FemX and LS174 cancer cell lines (Ranković et al., 2011). This cytotoxic activity is proposed to be associated with cell cycle arrest, modulation of reactive oxygen species balance, and activation of apoptotic pathways (Ranković et al., 2011). Furthermore, some *Cladonia*-derived metabolites have been reported to exert more selective effects on cancer cells compared with normal cells (Torres-Benítez et al., 2022). However, it has also been emphasized that the biological effects of *Cladonia* extracts are closely related to dose and extraction method; therefore, detailed toxicity and pharmacokinetic studies are required before therapeutic applications can be considered (Ranković et al., 2011).

5. Pharmaceutical, Cosmetic, and Biotechnological Importance

Due to their antioxidant, antimicrobial, and cytotoxic properties, *Cladonia* species are regarded as promising natural sources for pharmaceutical and cosmetic industries (Núñez-Arango et al., 2024). Particularly, *Cladonia*-derived metabolites exhibiting photoprotective and antioxidant effects are considered potential candidates for topical cosmeceutical formulations (Núñez-Arango et al., 2024). Compounds isolated from *Cladonia cf. didyma* collected from the Andes Mountains have demonstrated high SPF values and strong UVA/UVB protective capacity, highlighting the significance of these lichens as potential natural photoprotective agents (Núñez-Arango et al., 2024). Moreover, the identification of holobiont-based biosynthetic gene clusters provides an important foundation for the integration of *Cladonia* species into biotechnological production platforms (Shishido et al., 2021; Heuberger et al., 2025). In this context, *Cladonia* species are considered among

the lichen groups with high potential both for the discovery of new natural products and for sustainable production of biologically active compounds (Shishido et al., 2021).

Conclusion and Future Perspectives

The lichen genera *Usnea*, *Evernia*, *Parmelia*, *Ramalina*, and *Cladonia* discussed in this review clearly demonstrate the biochemical richness and multifaceted biological activity potential of lichens (Ingólfsdóttir, 2002). These genera possess a rich secondary metabolite profile, particularly in terms of depsides, depsidones, dibenzofuran derivatives, and various phenolic compounds; however, quantitative and qualitative variations may occur depending on species, habitat characteristics, and environmental conditions (Cansaran et al., 2006; Maulidiyah et al., 2023). This chemical diversity is considered one of the main determinants of differences in biological activity and provides important clues from a chemotaxonomic perspective (Elix & Stocker-Wörgötter, 2008). In terms of antioxidant activity, species belonging to *Usnea*, *Evernia*, *Parmelia*, *Ramalina*, and *Cladonia* exhibit pronounced free radical scavenging capacity, largely associated with phenolic compounds and specific secondary metabolites (Salgado et al., 2017; Sepahvand et al., 2021). In addition to findings obtained from in vitro methods such as DPPH, ABTS, and FRAP, data regarding DNA-protective effects indicate that lichen extracts can mitigate oxidative damage not only at the chemical level but also in biological systems (Sepahvand et al., 2021). This characteristic positions lichens as potential natural antioxidant sources that may be considered in the prevention or supportive treatment of oxidative stress–related diseases (Mitrović et al., 2011). Data on antimicrobial activity demonstrate that the five lichen genera evaluated possess significant inhibitory potential particularly against Gram-positive bacteria (Kınalıoğlu et al., 2019; Shcherbakova et al., 2021). Major contributors to this effect include usnic acid, atranorin, and related depsidone derivatives, which are thought to act by disrupting bacterial cell membrane integrity and suppressing metabolic processes (Ingólfsdóttir, 2002). Conversely, the relatively limited activity reported against Gram-negative bacteria is associated with resistance mechanisms related to their outer

membrane structure (Kosanić et al., 2013). These findings suggest that lichen-derived compounds may be more rationally utilized in targeted antimicrobial strategies rather than as broad-spectrum agents (Kosanić et al., 2013). Regarding anticancer and cytotoxic effects, extracts and purified metabolites obtained from species belonging to these five genera have been reported to inhibit cell proliferation, induce apoptosis, and exert regulatory effects on the cell cycle in various cancer cell lines (Aslan Engin et al., 2023). The selective toxicity of compounds such as usnic acid and certain phenolics toward cancer cells has attracted particular pharmacological interest (Guo et al., 2008). However, high-dose toxicity and uncertainties regarding safety profiles indicate the need for careful evaluation of dose, formulation, and targeting strategies before direct therapeutic applications are considered (Guo et al., 2008). Overall, species belonging to the genera *Usnea*, *Evernia*, *Parmelia*, *Ramalina*, and *Cladonia* represent promising natural biological resources for pharmaceutical, cosmetic, and biotechnological applications owing to their antioxidant, antimicrobial, and anticancer properties (Elix & Stocker-Wörgötter, 2008). Nevertheless, much of the current literature is still limited to in vitro studies, and the need for in vivo and preclinical investigations remains evident (Kello et al., 2023). From a future perspective, nanoformulation strategies, carrier systems, and targeted drug delivery approaches aimed at enhancing the bioavailability of lichen-derived bioactive compounds provide important directions for upcoming research (Kello et al., 2023). In addition, the application of metabolomics and multi-omics approaches to more comprehensively characterize species-specific metabolite profiles will significantly contribute to understanding the molecular mechanisms underlying biological activities (Maulidiyah et al., 2023). In this context, more detailed investigation of the relationship between ecological adaptations and biochemical characteristics of lichens emerges as an important research area for both basic sciences and applied biomedical studies.

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Chapter 4

CD9 IN HEALTH AND DISEASE: MOLECULAR ARCHITECTURE, BIOLOGICAL ROLES, AND EMERGING SIGNIFICANCE IN LEUKAEMIA AND SOLID TUMOURS

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1. Introduction

Cell membrane organisation is often mistaken as a passive arrangement of lipids and proteins, despite its compositional flexibility and lateral dynamics being modelled for more than a century (Singer & Nicolson, 1972). In reality, the cell surface constitutes a highly adjustable, coordinated landscape within which, interactions between receptors, scaffolding proteins, adhesion molecules, and signalling components dictate how cells perceive and respond to their microenvironment (Sarkar & Chattopadhyay, 2021; van Deventer et al., 2021). Among the critical architects of this functional architecture are the tetraspanins, a family of transmembrane proteins that associate laterally with a wide range of molecular partners to create dynamic platforms regulating adhesion, migration, signalling, and vesicle trafficking (Kummer et al., 2020; Rubinstein et al., 2025; Susa et al., 2024; Yunta & Lazo, 2003).

CD9, historically known as Motility-Related Protein-1 (MRP-1), or later, TSPAN29, is among the earliest identified members of the tetraspanin family (Maecker et al., 1997; Miyake et al., 1995). For decades it remained an enigmatic protein —widely expressed yet poorly understood —and was most often mentioned primarily as a surface marker (Boucheix & Benoit, 1988; Jankovičová et al., 2020). Accumulating evidence has since reframed CD9 as a potent regulator of intercellular communication and tumour–microenvironment interactions (Detchokul et al., 2014; Huan et al., 2015; Lorico et al., 2021; Ondruššek et al., 2023a). One of the most striking features of CD9 biology is its functional duality: in some cancers it appears to restrict migration and dissemination, whereas in others it enhances aggressiveness and theraputic resistance (Zöller, 2009). This functional diversity has prompted a re-evaluation of CD9 not merely as a biomarker but as a molecular contributor in cancer progression (De Bruyne et al., 2008; Guého et al., 2025; Gupta & Kumar, 2025; Lorico et al., 2021).

The reconsideration of CD9's importance has been especially pronounced surrounding haematological malignancies. In acute lymphoblastic leukaemia (ALL) (Guého et al., 2025) and acute myeloid leukaemia (AML) (Touzet et al., 2019), CD9 is increasingly recognised as a marker of leukemic stemness and a key mediator of blast interaction within the bone marrow niche (Nishida et al., 2009). The consistent expression of CD9 across many ALL subtypes also highlights its value in disease monitoring and early diagnostic assessment. Together, these observations raise fundamental questions about CD9 biology: How does it shape leukemic behaviour? Why are CD9-positive blasts often

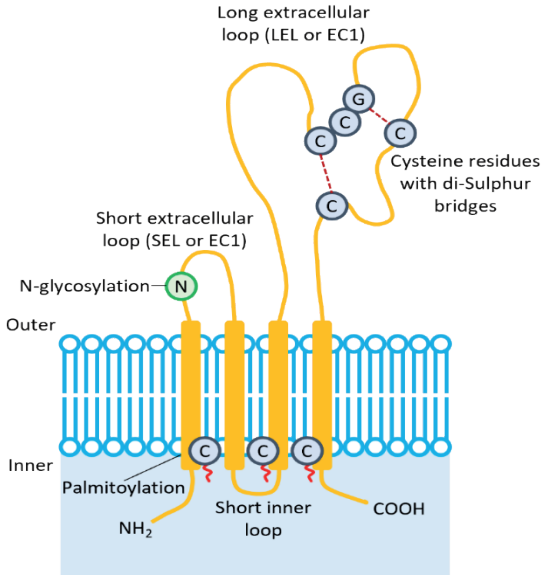
associated with increased migration and chemoresistance? And might therapeutic modulation of CD9 improve outcomes for patients with high-risk disease?

Addressing these questions requires considering CD9 not in isolation, but within the broader framework of the tetraspanin family. Accordingly, this chapter begins by outlining the fundamental biology of tetraspanins before exploring CD9's structure, regulation, and roles in both physiological and pathological contexts. It then explores how CD9 integrates environmental cues during haematopoiesis, how its expression patterns reshape leukemic behaviour, and why its clinical relevance continues to grow. Throughout, we build on the foundation of recent scientific literature, expanding the conceptual horizon to reflect the evolving understanding of this multifaceted protein.

2. Biology of Tetraspanins

Tetraspanins represent a family of transmembrane proteins defined by a conserved structure consisting of four membrane-spanning helices, two extracellular loops, and short cytoplasmic tails (Figure 1). This canonical blueprint remains largely preserved across the 33 human tetraspanin members (Stipp et al., 2003). In contrast, the extracellular loops, particularly the larger "EC2" domain, are believed to determine specificity in molecular interactions, whereas the intracellular regions orchestrate interactions with signalling and cytoskeletal components.

Figure 1: Classical tetraspanin protein family structure, including four membrane spanning domains, enabling fine control of membrane permeability. Membrane spanning domains are joined by extracellular loops of varying lengths which form structures depending on disulphide bridges between cysteine residues and post-translational modifications like N-glycosylations which together enable specific yet dynamic molecular interactions.



What distinguishes tetraspanins from other membrane proteins is their capacity to assemble into higher-order structures known as tetraspanin-enriched microdomains (TEMs). Within these lipid-protein assemblies, tetraspanins cluster with integrins, immunoglobulin-superfamily receptors, proteases, and signalling intermediates (Zuidscherwoude et al., 2015). These assemblies form organisational 'webs' that govern the localisation, interaction, and information transmission of associated proteins (Charrin et al., 2009). For example, integrin signalling becomes amplified or redirected depending on the tetraspanin composition of the microdomain, with clear consequences for cell adhesion and migration (Berditchevski, 2001).

Tetraspanins are ubiquitously expressed across tissues and regulate diverse processes including immune synapse formation, gamete fusion, neuronal connectivity, and epithelial barrier function (Hemler, 2005; Rubinstein, 2011). Their biological impact is rarely mediated through intrinsic enzymatic activity (Maecker et al., 1997), instead tetraspanins are modulated by the activity,

stability, and spatial distribution of other membrane proteins (Berditchevski, 2001). Palmitoylation of cytoplasmic cysteine residues is a key determinant of tetraspanin function, essential for stabilising TEMs, promoting tetraspanin-mediated lipid-rich membrane region anchoring, and supporting tetraspanin scaffolding functions (Charrin et al., 2009; Yang et al., 2004).

Within the tetraspanin family, certain members are known for particular physiological roles, CD81 in viral entry (Pileri et al., 1998), CD63 in secretory granules and exosomes (Andreu & Yanez-Mo, 2014; Escola et al., 1998; Jankovičová et al., 2020), CD82 as a metastasis suppressor (Tsai & Weissman, 2011), and CD37 in immune regulation (van Spriel et al., 2004). CD9 stands out as one of the most broadly studied due to its role in fertilization (Chen et al., 1999), haematopoiesis (Guého et al., 2025; Nishida et al., 2009), intercellular communication, and tumour progression (Detchokul et al., 2014; Huan et al., 2015; Miyake et al., 1995; Tsai & Weissman, 2011; Zöller, 2009).

3. CD9 Structure, Expression, and Regulation

CD9 is encoded on chromosome 12p13.31 and produces a 228-amino-acid protein that exhibits canonical tetraspanin domains (Kersey et al., 1981; Stipp et al., 2003; Susa et al., 2024). Its larger extracellular loop contains conserved cysteine residues that form disulfide bonds, maintaining tertiary structure and facilitating interactions with a range of partner proteins. These include integrins (Seigneuret et al., 2001), EWI family members (Stipp et al., 2001), metalloproteinases (particularly ADAM10/17) (Jouannet et al., 2016), and receptors associated with immune cell activation (Yeung et al., 2018). The variety of possible extracellular interactions help explain why CD9 influences adhesion and migration in many cell types.

Post-translational modifications shape CD9 behaviour at the membrane. Palmitoylation enhances its capacity to incorporate into TEMs (Yang et al., 2004), whereas N-glycosylation facilitates proper folding and protects the protein from proteolytic degradation (Charrin et al., 2009; Maecker et al., 1997). Notably, disruption of these regulatory mechanisms alters CD9's downstream associations with key partners and signalling outcomes (Charrin et al., 2009; Yang et al., 2004). CD9 expression is regulated at multiple levels including epigenetically. In certain cancers, such as multiple myeloma, neuroblastoma, and lung cancer, where DNA hypermethylation or histone

modifications reduce expression (De Bruyne et al., 2008; Hu et al., 2014; Yoon et al., 2010). In some tumour types, promoter demethylation via agents such as 5-aza-deoxycytidine restores CD9 expression, though the functional effects vary depending on tissue context.

Transcriptionally, CD9 is regulated by factors including Sp1, AP2, GRHL1, and hypoxia-responsive elements bound by HIF1A. The induction ofCD9 expression under hypoxic conditions provides a mechanistic explanation for its increased concentration within certain microenvironments such as bone marrow or extramedullary niches. Post-transcriptional regulation adds further complexity to CD9 expression (Gupta & Kumar, 2025): alternative 5′UTR variants influence translation efficiency, microRNAs such as miR-518f-5p target CD9 transcripts (Bond et al., 2020), and aberrant mRNA species lacking key domains have been observed in prostate cancer (J.-C. Wang et al., 2007). Together, these regulatory layers underscore that CD9 expression is neither static nor uniform, but instead reflects dynamic interactions between the genome, epigenome, microenvironment, and cellular stressors.

4. CD9 in Cell Adhesion, Migration, and Signal Modulation

CD9 is deeply integrated with the mechanisms that govern cell adhesion (Ondruššek et al., 2023b), migration (Hou et al., 2025; Yeung et al., 2018), and remodelling of their microenvironment (Huang et al., 2024; X. Liu et al., 2023). Its interactions with integrins such as α4β1 and α6β1 allow CD9 to fine-tune adhesion to extracellular matrix components, including fibronectin and laminin (Berditchevski, 2001; Gustafson-Wagner & Stipp, 2013; Hemler, 2005), (Figure 2A). Through these interactions, CD9 modulates the strength, duration, and spatial distribution of adhesive contacts (Berditchevski, 2001), a property particularly relevant to immune cells, stem cells, and cancer cells (Chastney et al., 2025).

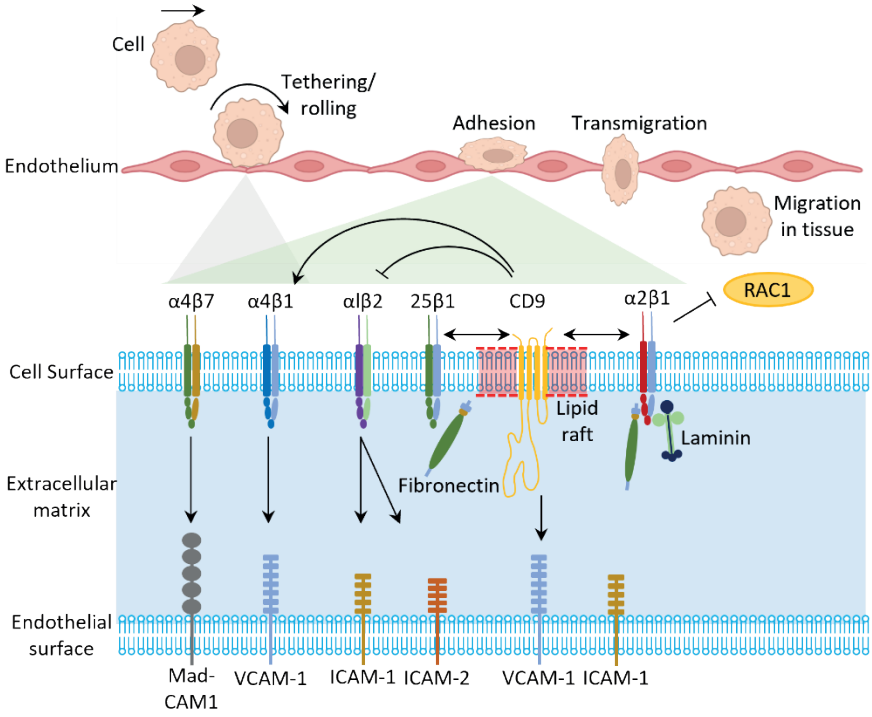


Figure 2: Integrated network of cell adhesion including cell tethering, adhesion, and migration, governed by tetraspanin proteins in concert with integrins on the cell's membrane and endothelial surfaces. Migration is inhibited by RAC1 in the absence of CD9.

One of CD9's best-documented functions is its ability to organise integrins and adhesion molecules into well-coordinated microdomains (Hemler, 2005; Kummer et al., 2020; Yunta & Lazo, 2003). On endothelial cells, CD9 contributes to the stabilisation of ICAM-1 and VCAM-1, which mediate leukocyte binding and diapedesis (Barreiro et al., 2005; Yeung et al., 2018), (Figure 2A). On leukocytes, CD9 enhances adhesion and promotes integrin activation during immune cell interactions and signalling events (Barreiro et al., 2005; Gustafson-Wagner & Stipp, 2013). CD9 influences cell migration by modulating actin polymerisation; engagement of CD9 can activate Rac1, promoting the formation of membrane protrusions that support motile behaviour (Figure 3) (Arnaud et al., 2015). Similarly, CD9 can modulate the activity of ADAM proteases, thereby influencing shedding of adhesion molecules or ECM degradation (Chen et al., 1999; Jouannet et al., 2016).

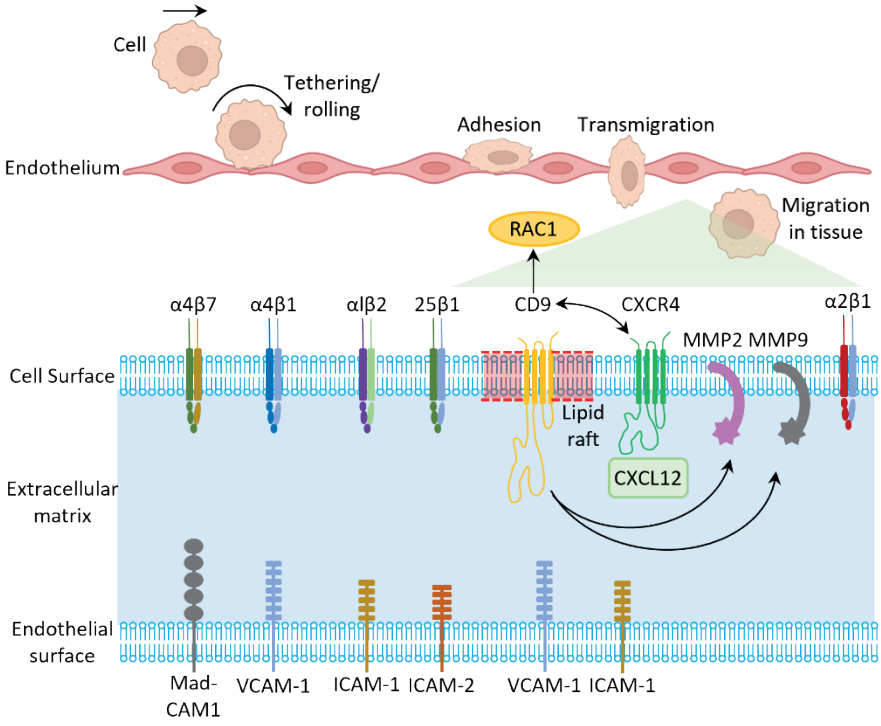


Figure 3: CD9 activation of RAC1 promotes motile behaviour. In turn, CD9 is upregulated by CXCL12 signalling, reinforcing the adhesive and migratory functions required for cancer cell localisation and persistence.

In cancer biology, these properties translate into context-dependent outcomes (Zöller, 2009). In some tumours, CD9 suppresses dissemination by stabilising adhesion structures and maintaining cell-cell/cell-matrix comminications (Higashiyama et al., 1995; Ikeyama et al., 1993). In contrast, CD9 enhances invasiveness in other tumour contexts by enabling cytoskeletal remodelling, matrix degradation, or chemokine-induced migration (Arnaud et al., 2015; Chastney et al., 2025; Hou et al., 2025). This functional dichotomy provides a conceptual framework for understanding why CD9 may be associated with either favourable or poor prognosis, depending on tumour type and microenvironmental context.

5. CD9 in Haematopoiesis

CD9 expression is dynamic during hematopoietic development. Hematopoietic stem cells (HSCs) display detectable but heterogeneous CD9 expression in early stages of commitment, with levels increasing during certain lineage transitions and decreasing during others (Jasim et al., 2025). In murine systems, long-term HSCs generally express higher CD9 levels than more committed progenitor populations, however CD9-deficient mice largely retain haematopoietic homeostasis, indicating CD9 is not solely responsible for maintaining steady state haematopoiesis (Le Naour et al., 2000; Miyado et al., 2000).

Within the bone marrow niche, CD9 contributes to the adhesion of HSCs and progenitors to stromal elements (Jasim et al., 2025). Through its interactions with integrins and chemokine receptors, CD9 influences how haematopoietic and leukemic cells respond to CXCL12, a central regulator of bone marrow homing and retention (Sugiyama et al., 2006), (Figure 3). In stromal niche contexts CXCL12 signalling can induce upregulation of CD9, reinforcing the adhesive and migratory functions required for localisation and persistence within the niche (Touzet et al., 2019).

CD9 also plays an important role in megakaryocyte maturation and platelet formation (Desterke et al., 2015). Engagement of CD9 on megakaryocytic progenitors influences downstream signalling pathways that regulate growth and differentiation (Boucheix & Benoit, 1988). In mature platelets, CD9 contributes to aggregation and signal amplification, underscoring its function extends beyond hematopoietic precursors to terminally differentiated lineages (Carroll et al., 1990; Qi et al., 1996; Worthington et al., 1990).

Collectively, these observations highlight CD9 as a mediator of stromal–hematopoietic interactions and as a contributor to the fine-tuned balance between quiescence, proliferation, and migration within the bone marrow microenvironment.

6. CD9 in Cancer: Dual Roles Across Tumour Types

One of the most striking features of CD9 is the variability of its role in cancer. Decades of accumulated evidence demonstrates that CD9 can serve as either

a metastasis suppressor or a promoter of invasiveness, depending on cellular context (Detchokul et al., 2014; Zöller, 2009).

In several solid tumours including melanoma (Fan et al., 2010), colorectal cancer (Kim et al., 2016), and squamous cell cancers (Higashiyama et al., 1995; Kusukawa et al., 2001), low CD9 expression correlates with advanced disease and increased metastatic potential. In these scenarios, CD9 appears to stabilise cell–cell interactions or maintain adhesive states incompatible with migration. Conversely, in breast cancer (Kwon et al., 2017), pancreatic cancer (V. M.-Y. Wang et al., 2019), and subsets of gastric carcinoma (Soyuer et al., 2010; Zeng et al., 2021), elevated CD9 expression has been associated with poorer outcomes, potentially reflecting its capacity to enhance motility or remodel the tumour microenvironment. Importantly, there are conflicting reports regarding CD9 expression and cancer prognosis across most types, highlighting the context dependency of its expression across cancers (Ondruššek et al., 2023a; Zöller, 2009). Mechanistically, CD9 can interact with metalloproteinases such as ADAM10 and ADAM17, thereby influencing cleavage of extracellular substrates and facilitating metastatic behaviour (Chen et al., 1999; Jouannet et al., 2016).

Part of CD9's functional ambiguity stems from the diversity of its molecular partners: association with specific integrins can reinforce adhesive states (Berditchevski, 2001; Hemler, 2005), whereas coupling to chemokine receptors and migratory signalling complexes may instead promote cell movement(Arnaud et al., 2015; Yeung et al., 2018). Moreover, CD9-containing extracellular vesicles can transfer pro-tumorigenic signals to neighbouring cells, influencing invasiveness, immune evasion, or niche colonisation (Bond et al., 2020; Hou et al., 2025).

Thus, CD9's role in cancer is not fixed but instead reflects an intersection between intrinsic cell programs and environmental cues. This complexity becomes even more evident in haematological malignancies, where microenvironmental factors strongly influence leukemic behaviour.

7. CD9 in Acute Leukaemia's

Acute leukaemia exemplifies the dualistic nature of CD9. In B-ALL, CD9 is highly expressed in a substantial portion of cases and has become recognised

as a diagnostically informative immunophenotypic biomarker (Leung et al., 2024; Touzet et al., 2019). Its expression can complement other immunophenotypic markers to refine risk stratification and assist in identifying cases with specific cytogenetic backgrounds.

High CD9 expression in ALL has often been linked to aggressive disease features. For example, CD9-positive blasts tend to display enhanced adhesion to bone marrow stromal elements and increased migratory potential toward chemokine gradients (Leung et al., 2024; Liang et al., 2018). Experimental models demonstrate that knocking down CD9 reduces invasive behaviour, decreases leukaemia burden in vivo, and increases sensitivity to certain chemotherapeutic agents (Y. Liu et al., 2021; Touzet et al., 2019).

A particularly significant aspect of CD9 biology in leukaemia relates to leukemic stem cell (LSC) behaviour. In AML models, CD9-positive blast subfractions are enriched for stem-like properties, including enhanced engraftment capacity and the ability to regenerate phenotypically heterogeneous disease in vivo (Y. Liu et al., 2021; Touzet et al., 2019). In acute lymphoblastic leukaemia, CD9 expression similarly marks blast populations with increased clonogenicity and stem-associated features, although functional in vivo evidence remains more limited (Nishida et al., 2009). These features align with stemness attributes: self-renewal, survival under stress, and resistance to therapy. Importantly, although CD9 can be detected on subsets of normal haematopoietic stem cells, its expression is often enriched, functionally amplified, or differentially regulated in leukemic stem cells, making CD9 a potentially useful marker for distinguishing malignant stem cell populations in specific disease contexts (Forsberg et al., 2005).

In AML, CD9 expression is more heterogeneous but still clinically informative. While several studies associate CD9 positivity with increased chemoresistance or aggressive disease (Touzet et al., 2019), others report longer survival in CD9-positive cohorts. These discrepancies mirror the complex biology of AML itself, which encompasses a highly diverse range of molecular subtypes and microenvironmental interactions. Collectively, CD9's roles in leukaemia extend from diagnosis to stem cell biology and treatment response, underlining its significance across the disease spectrum.

8. CD9 in Diagnosis, Prognosis, and MRD Monitoring

The diagnostic utility of CD9 is well established in B-ALL, where its high or moderate expression occurs across multiple cytogenetic subgroups (Wood, 2015). When combined with markers such as CD10, CD19, and CD34, CD9 helps identify leukemic blasts with improved precision (Wood, 2015). Its expression profile also assists in distinguishing subtypes such as ETV6-RUNX1-positive leukaemia, where lower CD9 levels are often observed (Blunck et al., 2019).

In APL, CD9 expression contributes to a characteristic immunophenotypic profile that aids in distinguishing APL from other HLA-DR–negative AML subtypes (Blunck et al., 2019; Gandemer et al., 2010; Ren et al., 2019). Given that rapid identification of APL is clinically critical due to the risk of coagulopathy, CD9 adds value in early diagnostic decision-making. From a prognostic perspective, high CD9 expression has been associated with poorer survival in several large paediatric ALL cohorts (Leung et al., 2024). Notably, these correlations persist even after accounting for minimal residual disease and other risk factors, suggesting that CD9 may mark a more aggressive biological phenotype. Meanwhile, the relative stability of CD9 expression across treatment courses further supports its utility in minimal residual disease monitoring which can be achieved with by flow cytometry (Dong et al., 2011; Wood, 2015). Its consistent presence on residual leukemic blasts provides a phenotypic anchor for tracking disease persistence even when other markers fluctuate. Collectively, these diagnostic and prognostic roles highlight CD9 as a reliable and informative marker throughout the clinical course of leukaemia.

9. CD9 in Therapeutic Targeting

Given its surface expression and role in leukemic biology, CD9 has become an appealing candidate for therapeutic intervention. Preclinical studies, primarily in acute myeloid leukaemia models, indicate that antibody-mediated targeting or functional suppression of CD9 reduces migratory and niche-associated behaviour, decreases leukemic burden in murine xenografts, and enhances responsiveness to conventional chemotherapeutic agents (Y. Liu et al., 2021; Touzet et al., 2019). These results suggest that CD9 contributes

directly to leukaemia progression and that its inhibition may disrupt critical interactions between blasts and microenvironmental niches.

However, targeting CD9 is not without challenges. CD9 is widely expressed on normal tissues, including platelets, where antibody-mediated engagement can trigger activation. Early studies of anti-CD9 antibody candidates reported induced platelet aggregation, creating safety concerns for clinical translation (Lorico et al., 2021; Worthington et al., 1990). Despite this, newer antibody formats and engineering strategies—such as Fc-modified antibodies, bispecific antibodies, or antibody fragments—may offer safer and more selective targeting options (Goldmann et al., 2019; Schotte et al., 2020).

Beyond antibody-based approaches, CD9 may be targeted indirectly by modulating pathways that regulate its expression, including epigenetic modulators or signalling inhibitors (De Bruyne et al., 2008; Ondruššek et al., 2023a). Alternatively, CD9-positive extracellular vesicles could be exploited as diagnostic tools or therapeutic targets, given their involvement in intracellular communications and disease propagation (Khalife et al., 2020; Rubinstein et al., 2025). As understanding of CD9's contributions to leukaemia continue to deepen, the prospect of incorporating CD9 into multi-modal therapeutic strategies becomes increasingly compelling, with potential to weaken leukemic stemness, disrupt niche interactions, and improve long-term treatment outcomes.

10. Future Perspectives

The study of CD9 has entered a new phase in which traditional views of membrane markers are giving way to more nuanced interpretations of molecular function. The growing recognition of CD9 as a regulator of stemness, microenvironmental crosstalk, and treatment resistance invites further exploration across multiple fronts.

Future research will likely focus on dissecting the specific molecular partners that dictate CD9's varied effects. Understanding why CD9 promotes aggressiveness in certain tumours while suppressing metastasis in others will require systematic mapping of the protein complexes it forms under different

biological conditions. High-resolution imaging, proximity labelling, and single-cell profiling are expected to be instrumental in these efforts.

In leukaemia, elucidating how CD9 influences homing to bone marrow niches or extramedullary sites may yield actionable therapeutic insights. Likewise, integrating CD9 expression patterns into risk stratification has the potential to refine prognostic accuracy and facilitate personalised therapeutic strategies. As targeted therapies and immunotherapies continue to reshape the landscape of leukaemia treatment, CD9 may emerge as a complementary target either directly, through antibody-based inhibition, or indirectly, by manipulating signalling pathways associated with CD9-rich microdomains.

Ultimately, CD9 occupies a critical intersection between fundamental cell biology and translational oncology. Its capacity to influence diverse signalling networks positions it as a compelling subject for continued investigation with the potential to inform not only on leukaemia, but also into the broader architecture of cellular communication in health and disease.

Conclusion

CD9 is far more than a surface marker, it is a molecular integrator that shapes how cells interact with their environment, how they organise their membrane networks, how they migrate, proliferate, and respond to stress. Its functions span physiological processes such as haematopoiesis and immune regulation, but its roles in pathology especially in cancer have captured increasing attention.

In acute leukaemia, CD9 bridges diagnostic utility, prognostic significance, and biological influence. It helps define leukemic stem cell behaviour, facilitates microenvironmental communication, and contributes to the migratory and adhesive properties that allow blasts to colonize various tissues. The complexity of CD9's actions offers both challenges and opportunities for therapeutic intervention.

Understanding CD9 within the broader context of tetraspanin biology will continue to illuminate the intricacies between the interplay of membrane organisation, signalling, and environmental adaptation that underpins cancer

progression. As research advances, CD9 will undoubtedly remain a central figure in discussions of leukaemia biology and may ultimately serve as a bridge between fundamental cellular science and clinically impactful therapies.

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Chapter 5

METFORMIN AND OXIDATIVE STRESS: MOLECULAR MECHANISMS, PROTECTIVE EFFECTS ON THE RETINA, AND THERAPEUTIC PERSPECTIVES

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Introduction

Diabetes mellitus, whether type 1 or type 2, is a chronic and systemic metabolic disorder characterized by hyperglycemia that arises from insufficient insulin secretion by pancreatic β-cells or from resistance to insulin action in peripheral tissues (Ağgül, 2012; TEMD, 2022). While type 1 diabetes is marked by autoimmune β-cell destruction, type 2 diabetes is characterized by the coexistence of progressive decline in insulin secretion and insulin resistance (ElSayed et al., 2023; Rewers et al., 2015; Kothari et al., 2016). Approximately 90-95% of individuals with diabetes worldwide have type 2 diabetes, the etiology of which is associated with complex interactions between multiple genetic and environmental factors that are not yet fully understood (Desu et al., 2005; Kothari et al., 2016).

Diabetic retinopathy (DR) is one of the most common and most devastating microvascular complications of diabetes, ranking among the leading causes of blindness globally (Hendrick et al., 2015). The pathological processes occurring in the retina are driven primarily by hyperglycemia-induced oxidative stress, inflammation, endothelial dysfunction, and aberrant angiogenesis. Epidemiological data indicate that DR has a high prevalence in both type 1 and type 2 diabetes, and results from large population-based studies demonstrate that the lifetime risk exceeds 50% (Klein, 2009; Sabanayagam et al., 2019).

It has long been recognized that hyperglycemia exacerbates oxidative stress and microvascular injury. The UKPDS (United Kingdom Prospective Diabetes Study) demonstrated that glycemic control directly influences microvascular damage and that each incremental rise in blood glucose levels increases the risk of retinopathy (Stratton et al., 2000). Therefore, effective glycemic management and reduction of oxidative stress are critical for preventing DR and slowing its progression.

Oxidative stress is a biochemical condition that arises from an imbalance between reactive oxygen species (ROS), which are intrinsic components of

cellular physiology, and the antioxidant defense mechanisms that neutralize them. Under normal physiological conditions, mitochondrial respiration, NADPH oxidase activity, and various enzymatic reactions generate limited amounts of ROS; these molecules play important roles in cellular signal transduction, regulation of immune responses, and maintenance of homeostasis. However, when ROS production increases or antioxidant defenses become insufficient, pathological processes such as lipid peroxidation, protein oxidation, DNA damage, mitochondrial dysfunction, and inflammation ensue (Betteridge, 2000; Liguori et al., 2018). The damage inflicted by oxidative stress on cellular components plays a pivotal role in the progression of chronic conditions including aging, neurodegenerative diseases, cardiovascular pathologies, and particularly diabetes.

Chronic hyperglycemia in diabetes mellitus is the most significant pathophysiological factor contributing to increased oxidative stress. Hyperglycemia enhances superoxide production through excessive electron flux within mitochondria, accelerates NADPH consumption by activating the polyol pathway, and strengthens inflammation by triggering RAGE signaling via advanced glycation end products (AGEs). These pathological mechanisms together form a common "oxidative stress and inflammation axis" that underlies the development of diabetic complications (Brownlee, 2001; Giacco & Brownlee, 2010). In complications such as retinopathy, nephropathy, and neuropathy, the accumulation of ROS disrupts cellular signaling networks, leading to endothelial dysfunction, apoptosis, and impairments in tissue integrity. The retina is particularly vulnerable to oxidative damage due to its high oxygen consumption, rich mitochondrial content, and energy-demanding phototransduction processes (Kowluru, 2001).

Metformin, one of the primary oral antidiabetic agents recommended as first-line therapy for type 2 diabetes, exerts glycemic control mainly by reducing hepatic gluconeogenesis and enhancing insulin sensitivity in peripheral tissues (Bailey, 2015; Chaudhury et al., 2017). However, translational and molecular studies in recent years have revealed that metformin extends far beyond being

merely a glucose-lowering agent and exhibits a wide array of protective effects at the cellular level.

This broad biological activity of metformin has accelerated research into its potential role in the pathogenesis of diabetic retinopathy, one of the major microvascular complications of diabetes. Hyperglycemia-induced oxidative stress, inflammation, and pathological angiogenesis in retinal tissue play critical roles in the progression of DR. Metformin modulates these processes through multiple mechanisms, including AMPK activation, regulation of mitochondrial functions, reduction of ROS production, suppression of NF-κB–mediated inflammatory responses, and modulation of the VEGF signaling pathway (Han et al., 2018; Yi et al., 2016).

Furthermore, metformin strengthens antioxidant systems and protects key cellular functions, such as ion homeostasis, protein stability, and stress-response pathways, by activating the Nrf2–HO-1 axis (Oubaha et al., 2016). Additionally, its ability to reduce microglial activation, prevent vascular leakage, and inhibit endothelial cell proliferation and migration underscores its antiangiogenic and neuroprotective properties, thereby enhancing its therapeutic potential in retinal diseases (Han et al., 2018).

Recent preclinical and clinical studies have demonstrated that metformin can preserve the structural and functional integrity of the retina by decreasing oxidative stress, suppressing inflammation, and modulating cell death pathways. Experimental models have shown that metformin increases the expression of antioxidant enzymes in retinal ganglion cells, reduces lipid peroxidation, and suppresses pro-apoptotic signaling (Amin et al., 2022). Clinical observations further indicate significantly reduced incidence and progression of diabetic retinopathy in diabetic patients using metformin (Chen et al., 2019). These findings support the consideration of metformin as a potential “retinoprotective agent” for the prevention and management of oxidative stress, based complications such as diabetic retinopathy.

In light of this evidence, metformin has gained increasing attention due to its low cost, favorable safety profile, and pleiotropic biological effects, particularly in attenuating oxidative stress, mediated retinal damage or slowing the development of diabetic retinopathy. Both experimental and clinical data suggest a protective role of metformin on the diabetic retina and highlight the need to evaluate this drug for novel therapeutic applications beyond its traditional use (Bailey, 2015; Han et al., 2018).

This section provides a detailed examination of metformin's effects on oxidative stress, the associated molecular pathways, the biological responses of the retina to oxidative burden, and the therapeutic potential demonstrated in preclinical and clinical studies. In doing so, it offers a comprehensive assessment of the metformin, oxidative stress interaction from both basic science and clinical perspectives.

1.The Role of Oxidative Stress in Diabetic Retinopathy

Excessive production of ROS, mitochondrial dysfunction, accumulation of AGEs, impaired nitric oxide synthesis, and increased activation of NADPH oxidase play major roles in the development of diabetic retinopathy (Mohamed et al., 2007). Oxidative stress leads to structural and functional alterations in retinal endothelial cells, pericytes, photoreceptors, Müller glia, and neuronal cells.

Recent translational studies have demonstrated that aging, cellular senescence, and inflammation progress concurrently within the retina, and that these processes are accelerated by oxidative stress (Oubaha et al., 2016). These findings highlight oxidative stress as a central therapeutic target in the pathophysiology of DR.

2. Molecular Mechanisms of Metformin Action

2.1. AMPK Activation

Metformin activates AMPK pathways, which regulate cellular energy homeostasis. AMPK activation contributes to the reduction of oxidative stress, enhancement of mitochondrial biogenesis, and suppression of inflammatory signaling (Bailey, 2015). In the retina, AMPK activation has been associated with neuroprotective effects.

2.2. Modulation of Mitochondrial Complex I

Metformin reduces the activity of mitochondrial complex I, thereby lowering ATP production and increasing the AMP/ATP ratio. This shift helps restore energy balance and limits ROS generation (Bailey, 2015; Chaudhury et al., 2017).

2.3. Suppression of Inflammatory Signaling

Metformin decreases inflammatory markers such as NF-κB, TNF-α, and IL-6, contributing to the attenuation of retinal inflammation (Han et al., 2018).

2.4. Regulation of Angiogenesis

Metformin has been shown to modulate VEGF signaling, particularly by altering alternative splicing of VEGF-A, thereby reducing pathological angiogenesis (Yi et al., 2016).

3. Antioxidant Effects of Metformin

Metformin reduces oxidative stress at both systemic and local (retinal) levels. Its major antioxidant effects can be summarized as follows:

- Reduction of ROS production by decreasing NADPH oxidase activity,
- Enhancement of antioxidant enzyme levels (SOD, catalase, GPx),
- Reduction of toxic glucose-derived by-products (AGEs, PKC activation),

- Stabilization of mitochondrial function.

As a result of these mechanisms, several studies have reported decreased oxidative damage in retinal vasculature and neural tissue (Han et al., 2018; Yi et al., 2016).

4. Protective Effects on the Retina

4.1. Neuroprotective Effects

One of the major benefits of metformin is the reduction of oxidative stress–induced apoptosis in retinal neurons. AMPK activation and mitochondrial protection constitute the key mechanisms underlying this process (Han et al., 2018).

4.2. Vascular Protection

Metformin strengthens endothelial barrier function, reduces pericyte loss, and suppresses abnormal vascular leakage. Its ability to lower VEGF levels serves as an important protective mechanism during the proliferative stages of retinopathy (Yi et al., 2016).

4.3. Reduction of Inflammation

The suppression of pro-inflammatory mediators produced by Müller cells and microglia contributes to decreased inflammation within retinal tissue (Han et al., 2018).

4.4. Inhibition of Angiogenesis

Both in vitro and in vivo studies have demonstrated that metformin significantly reduces pathological angiogenesis (Han et al., 2018; Yi et al., 2016).

5. Metabolic Control and Clinical Findings

Metformin is recommended as first-line therapy for type 2 diabetes and plays an important role in reducing long-term microvascular complications (Zoungas et al., 2017). Large-scale meta-analyses have shown that intensive glycemic control lowers the risk of retinopathy (Zoungas et al., 2017; Stratton et al., 2000).

Although clinical studies directly evaluating the effects of metformin on retinopathy development are limited, it is widely accepted that metformin provides indirect protection by reducing glycemic burden. Furthermore, network-based drug comparison analyses have shown that metformin does not increase the risk of diabetic retinopathy and is safer than some other agents (Tang et al., 2018).

6. Therapeutic Perspectives

Considering its antioxidant, anti-inflammatory, and anti-angiogenic effects on the retina, metformin may represent a strong therapeutic candidate for future applications:

- Complementary agent in oxidative stress–targeted therapies,
- Supportive medication in combined anti-VEGF treatment protocols,
- Adjunctive agent in neuroprotective therapeutic strategies,
- Potential utility in reducing age-related retinopathy risk in non-diabetic individuals.

However, long-term randomized clinical trials are necessary before these applications can be recommended in clinical practice.

Conclusion

Metformin is not merely a drug that provides glycemic control; it is a multifaceted biological modulator with demonstrated effects in reducing oxidative stress, suppressing inflammation, inhibiting abnormal angiogenesis, and promoting cellular protection. These properties are directly linked to the fundamental processes underlying the pathophysiology of diabetic retinopathy.

Current evidence indicates that metformin can exert significant protective effects on retinal tissue and may hold potential as a complementary therapeutic agent in the future.

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Chapter 6

FROM PLANT EXTRACTS TO DRUG DISCOVERY: INTEGRATING OMICS TECHNOLOGIES

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1. General Definition of Omics Science

Omics science refers to the systematic and large-scale analysis of biological molecules that collectively define the structure, function, and dynamic behaviour of a biological system at a given organizational level. By enabling comprehensive data acquisition across different molecular layers, omics technologies have fundamentally reshaped how biological questions are formulated and investigated. Rather than relying on reductionist strategies that focus on isolated components, modern omics approaches integrate top–down, data-driven methodologies with bottom–up experimental designs, thereby allowing biological systems to be examined as interconnected and dynamic entities. The emergence of omics-based frameworks has been particularly transformative in the study of complex diseases such as cancer. Traditional low-throughput approaches, which primarily distinguished malignant tissues from healthy counterparts in a static manner, have been progressively replaced by global and unbiased analytical strategies. These new paradigms capture molecular heterogeneity across genomic, transcriptomic, proteomic, and metabolomic layers while also providing spatial and temporal resolution of disease-associated alterations. Since the introduction of DNA microarrays as the first generation of high-throughput platforms, omics technologies have undergone rapid technological evolution, expanding both in analytical depth and biological scope. Aligned with the central dogma of molecular biology, genomics has enabled the identification of stable genetic variations, whereas transcriptomics has revealed time-dependent fluctuations in gene expression. Proteomics has further extended this framework by uncovering protein abundance changes, post-translational modifications, and spatial dynamics that cannot be inferred solely from nucleic acid–based data. Beyond these core domains, the omics landscape has expanded to include epigenomics, epitranscriptomics, and epiproteomics, which collectively describe regulatory modifications that extend beyond primary sequence information within individual cells. In parallel, the development of interactome-level analyses, as well as metabolomics and immunomics, has provided additional layers of information relevant to disease mechanisms and biomarker discovery. The integration of multiple omics layers has therefore become a central strategy for linking molecular signatures to phenotypic outcomes. By combining heterogeneous datasets within a unified analytical framework, multi-omics approaches facilitate the identification of causal

relationships that would remain obscured in single-layer analyses. Recent advances in single-cell sequencing technologies have further enhanced this capability, enabling molecular profiling at cellular resolution and revealing previously unrecognized sources of biological variability. Despite these advances, the continuous expansion of omics technologies has also highlighted the intrinsic complexity of cellular systems. As new molecular layers and regulatory mechanisms are uncovered, existing models of cellular organization are frequently revised. Although omics-driven research has brought biological systems closer to comprehensive characterization, it simultaneously underscores the challenges associated with achieving complete control over pathological processes. Consequently, a critical reassessment of current achievements, limitations, and future directions in omics research is necessary to identify efficient pathways toward translational and clinical applications (Dai & Shen, 2022). In medical research, the increasing adoption of omics-based methodologies has been facilitated by advances in high-throughput data generation, bioinformatics infrastructure, and data storage capacity. Nevertheless, despite their extensive use in experimental settings—exemplified by large-scale applications during the COVID-19 pandemic—the routine clinical implementation of omics technologies remains confined to a limited number of disciplines (D'Adamo et al., 2021).

2. Drug Discovery Studies

2.1. Drug Targets

Drug discovery research is fundamentally driven by the identification of biological entities whose modulation can alter disease progression in a predictable and therapeutically beneficial manner. Among these entities, proteins have historically constituted the principal class of drug targets, as they participate directly in cellular signalling, metabolism, structural organization, and regulatory control. An ideal drug target is typically embedded within a disease-relevant biological pathway and must be sufficiently characterized at both functional and structural levels to allow rational therapeutic intervention. From a molecular perspective, this generally requires the presence of a defined binding region capable of accommodating drug-like compounds. Early drug discovery strategies relied heavily on structural analyses to determine whether a protein could interact with small molecules, leading to the formulation of the concept of *druggability*. A druggable target

is commonly described as a macromolecule whose three-dimensional conformation permits stable and specific binding to chemically tractable ligands. However, structural compatibility alone does not guarantee therapeutic success. In many cases, ligand binding does not translate into meaningful biological modulation or clinical benefit, highlighting the limitations of druggability as a sole selection criterion. Over the past two decades, systematic efforts have been undertaken to curate and classify therapeutic targets based on accumulated pharmacological evidence. Within large-scale resources such as the Drug Target Database (DTD), certain protein families are disproportionately represented, including proteases, kinases, G protein–coupled receptors (GPCRs), and nuclear hormone receptors. This enrichment reflects both their central roles in disease-associated signalling pathways and their amenability to pharmacological modulation. Nevertheless, contemporary target identification strategies extend beyond structural considerations and increasingly emphasize the functional relevance of candidate targets within disease-specific molecular networks. From a mechanistic standpoint, drug targets can be categorized according to their mode of action. Imming et al. (2006) proposed a broad classification encompassing enzymes, receptors, ion channels, transporters, nucleic acids, ribosomes, substrates, monoclonal antibody targets, and regulatory proteins. Although proteins continue to dominate therapeutic development pipelines, recent advances have expanded the target landscape to include nucleic acid–based structures. Regulatory DNA elements and non-coding RNAs (ncRNAs), in particular, have emerged as promising therapeutic targets due to their central roles in gene expression control. The growing interest in RNA-targeted therapeutics reflects the multifunctional nature of RNA molecules in cellular biology. Beyond their classical roles in transcription and translation, RNA species are involved in catalytic activity, intracellular trafficking, post-transcriptional regulation, and RNA processing events. Compared to DNA, RNA exhibits greater structural plasticity and lacks robust repair mechanisms, features that can be exploited for selective therapeutic intervention (Paananen & Fortino, 2020). The ability of RNA molecules to adopt complex three-dimensional conformations enables highly specific molecular interactions, facilitating the development of RNA-directed drugs for antibacterial, antiviral, and anticancer applications. The expanding catalog of ncRNAs has further revealed that gene regulation operates through multi-layered and interconnected networks. While this complexity offers new therapeutic opportunities, it also presents significant challenges. The functional roles of

many ncRNA species remain incompletely characterized, complicating efforts to translate basic discoveries into clinical applications. Consequently, ncRNA-based drug development is widely regarded as a high-potential yet technically demanding frontier in modern pharmacological research (Sah & Aronin, 2011).

2.2. The Significance of Ethnopharmacological Research in Pharmaceutical Development: The Contribution of Medicinal Plants

Interest in plant-derived therapeutics has persisted throughout human history, forming the empirical foundation of many traditional medical systems. Although synthetic drugs often provide rapid and targeted therapeutic effects, their use is frequently accompanied by substantial limitations, including adverse side effects, high production costs, and restricted accessibility in economically disadvantaged regions. In contrast, medicinal plants and herbal preparations are generally perceived as more accessible treatment options, often associated with improved biocompatibility and metabolic tolerance within the human body (Nasim et al., 2022).Archaeological and historical evidence indicates that the medicinal use of plants dates back several millennia. One of the earliest documented records, a Sumerian clay tablet discovered in Nagpur and dated to approximately 3000 BCE, describes the therapeutic application of plant-based remedies. Comparable records from Mesopotamian, Egyptian, Greek, and Islamic civilizations further demonstrate the widespread and systematic use of medicinal plants across ancient cultures (Petrovska, 2012). These early pharmacopeia's document the therapeutic use of nearly one thousand plant species, including representatives such as, *Papaver somniferum, Glycyrrhiza glabra Commiphora myrrha*, *Cupressus sempervirens* and *Cedrus* spp.(Borchardt, 2002). During these early periods, medicinal preparations were predominantly administered in crude forms, including powders, decoctions, infusions, tinctures, and topical poultices. The transition from traditional herbal medicine to modern pharmacology began with the isolation of bioactive compounds from plant sources in the early nineteenth century. A pivotal milestone was achieved in 1803 when Friedrich Serturner successfully isolated morphine from opium, establishing the scientific basis of natural products chemistry. This breakthrough was followed by the isolation of numerous pharmacologically active compounds, including strychnine from *Strychnos nux-vomica,* emetine from *Carapichea ipecacuanha*, colchicine

from *Colchicum autumnale*, quinine from *Cinchona officinalis*, papaverine from *Papaver somniferum* salicin from some *Salix* species, and atropine from *Atropa belladonna* (Allen & Hatfield, 2004; Siddiqui et al., 2014).The availability of purified natural compounds enabled a paradigm shift in Western medicine by allowing the controlled administration of defined chemical entities rather than complex crude extracts. This development significantly improved dosing accuracy, safety, and therapeutic efficacy, thereby marking the emergence of the modern pharmaceutical era (Süntar, 2020). As a result of their diverse pharmacological activities, medicinal plants have continued to provide substantial economic and societal value. However, the growing global demand for plant-based therapeutics has also intensified pressures on natural populations, leading to an increased risk of species depletion driven by habitat loss and unsustainable harvesting practices (Wang et al., 2020).Unlike conventional agricultural crops, medicinal plants present unique challenges for breeding and large-scale cultivation. The complexity of these species arises from the need to simultaneously optimize biomass production, growth cycles, and the accumulation of bioactive metabolites within specific plant organs. Traditional breeding approaches are often insufficient to address these multifactorial requirements. In this context, the integration of multi-omics analyses with advanced bioinformatics and statistical frameworks offers a powerful strategy for dissecting the genetic, biochemical, and regulatory bases underlying metabolite diversity and pharmacological activity in medicinal plants. Comprehensive elucidation of the genes, enzymes, and regulatory networks involved in the biosynthesis of therapeutically relevant metabolites provides a foundation for the application of genome engineering and synthetic biology approaches. Such strategies hold significant promise for the controlled, scalable, and sustainable production of high-value bioactive compounds. Consequently, the incorporation of multi-omics datasets into medicinal plant research should be regarded as a strategic priority and actively supported by international research institutions to meet the pharmaceutical industry's growing demand for natural product–derived therapeutics (Zhang et al., 2023).

2.3. Omics Applications for Drug Target Discovery Studies

High-throughput omics technologies constitute a central component of contemporary drug discovery pipelines, providing systematic insights into the molecular architecture of diseases and therapeutic responses. Genomics,

proteomics, and metabolomics are routinely applied across multiple stages of drug development, ranging from early target identification to late-stage efficacy and toxicity assessment. Among these, genomics has played a particularly transformative role with the advent of next-generation sequencing (NGS) platforms, which have enabled rapid, large-scale interrogation of genetic variation and gene expression landscapes. Genomic analyses facilitate the identification of disease-associated variants, transcriptional dysregulation, and pathway-level perturbations by enabling comparative assessments between healthy and diseased tissues. Such approaches have proven valuable not only for uncovering novel drug targets but also for predicting adverse drug reactions and stratifying patient populations based on molecular profiles. Expression-based genomic data, in particular, provide functional context by linking genetic alterations to downstream biological consequences. Proteomics complements genomic information by directly capturing changes at the protein level, where most therapeutic interventions exert their effects. Advances in mass spectrometry–based proteomic methodologies have enabled the quantitative and qualitative analysis of complex protein mixtures with high sensitivity. Techniques such as; isotope-coded affinity tagging (ICAT), isobaric labelling strategies (iTRAQ/TMT) stable isotope labelling with amino acids in cell culture (SILAC) and multidimensional protein identification technology (MudPIT) allow precise comparisons of protein abundance across biological conditions. In addition, functional proteomic approaches—including activity-based probes, protein and peptide arrays, phage display systems, and yeast two-hybrid assays—provide critical insights into protein–protein interactions, enzyme activity, and target engagement during drug development. Although metabolomics is comparatively novel than genomics and proteomics, it contributes uniquely to drug target discovery by capturing the end-point biochemical consequences of genetic and proteomic regulation. Metabolomic profiles reflect dynamic changes in cellular physiology and are particularly informative for elucidating drug mechanisms of action and metabolic liabilities. The continued maturation of metabolomics platforms, together with their integration with upstream omics layers, is expected to substantially enhance the identification and validation of therapeutically relevant targets (Russell et al., 2013).Technological advances in sequencing, microarray platforms, and mass spectrometry have collectively enabled the generation of multi-dimensional omics datasets at unprecedented resolution. These datasets provide comprehensive molecular snapshots that can be interrogated to

identify candidate drug targets, decode signalling networks, and evaluate potential safety concerns associated with pharmacological interventions. Importantly, omics-driven approaches also form the molecular basis of personalized medicine. Accumulating evidence demonstrates that genetic and molecular variability among individuals can significantly influence drug efficacy and toxicity profiles, underscoring the need for patient-specific therapeutic strategies. When systematically integrated, omics-derived molecular signatures associated with disease states and drug exposure can dramatically accelerate drug discovery and development processes. Integrative analyses enable the prioritization of targets with higher translational potential and reduce attrition rates by identifying failure risks at early stages. In this context, curated plant- and omics-oriented databases provide indispensable resources by organizing genomic, transcriptomic, proteomic, and metabolomic information in a form accessible for computational and experimental drug discovery efforts. The main omics approaches and plant databases are presented in Table 1.

Table 1. Omics and some plant databases

Omics Category	Database	Release Year	Reference
Genomic	WP-MOD	2025	(Wang et al., 2025)
	AMIR	2025	(Liu et al., 2025)
	MetaDB	2024	(Gao et al., 2024)
	MPOD	2022	(He et al., 2022)
	TCMPG	2022	(Meng et al., 2022)
	1K-MPGD	2022	(Su et al., 2022)
	GPGD	2021	(Liao et al., 2021)
	GWH	2021	(M. Chen et al., 2021)
	BPGD	2021	(Zhou et al., 2021)
	HMOD	2018	(Wang et al., 2018)
	Assembly	2016	(Kitts et al., 2016)

	Phytozome	2012	(Goodstein et al., 2012)
	PlantGDB	2004	(Brendel, 2004)
	EnsemblPlants	2002	(Hubbard et al., 2002)
Transcriptomic	PlantExp	2023	(Liu et al., 2023)
	MPOD	2022	(He et al., 2022)
	PPRD	2022	(Yu et al., 2022)
	PCMDB	2022	(Jin et al., 2022)
	BPGD	2021	(Zhou et al., 2021)
	PsctH	2021	(Xu et al., 2021)
	PlantscRNAdb	2021	(H. Chen et al., 2021)
	GPGD	2021	(Liao et al., 2021)
	HMOD	2018	(Wang et al., 2018)
	PlantGenIE	2015	(Sundell et al., 2015)
	Genevestigator	2008	(Hruz et al., 2008)
	ePlant	2005	(Toufighi et al., 2005)
Proteomic	PlantPReS	2016	(Mousavi et al., 2016)
	PPDB	2004	(Sun et al., 2009)
Metabolomic	PMN	2021	(Hawkins et al., 2021)
	HMOD	2018	(Wang et al., 2018)
	MetaCrop	2008	(Grafahrend-Belau et al., 2007)

2.4. Incorporating high-throughput omics technologies into medicinal plant research

Plant-derived natural products, including alkaloids, terpenoids, polyphenols, coumarins, and saponins, have attracted increasing interest from the pharmaceutical industry due to their broad spectrum of biological activities, such as antimicrobial, antioxidant, antidiabetic, anti-inflammatory and anticancer properties (Mumtaz et al., 2017). Despite their therapeutic potential, the large-scale and sustainable production of these compounds has historically been constrained by limited genetic and molecular information available for medicinal plant species. The rapid development of high-throughput sequencing technologies has begun to overcome these limitations by enabling the generation of high-quality genomic resources for a growing number of medicinal plants. The availability of whole-genome sequences has facilitated the functional characterization of genes involved in specialized metabolite biosynthesis. In several cases, elucidation of biosynthetic pathways has only become possible after genomic data were made accessible. For example, following the sequencing of the *Panax notoginseng* genome, genes responsible for the biosynthesis of saponins—compounds known for their antimicrobial and anti-inflammatory properties—were identified and functionally characterized, enabling their heterologous expression in alternative biological systems. Similarly, genomic insights into *Papaver somniferum* have clarified the molecular framework governing the biosynthesis, regulation, and intracellular transport of benzylisoquinoline alkaloids, a class of pharmaceutically important metabolites. The integration of additional high-throughput omics layers, including transcriptomics, proteomics, and metabolomics, has further expanded the analytical power of medicinal plant research. Combined omics analyses enable the identification of key regulatory genes, enzymes, and metabolites associated with pharmacological traits. For instance, coordinated transcriptomic and metabolomic profiling of *Podophyllum hexandrum*, a plant known for producing anticancer lignans, led to the identification of genes involved in the podophyllotoxin biosynthetic pathway. In another example, transcriptome-based investigations of *Abelmoschus esculentus* revealed genetic markers linked to anthocyanin accumulation and fruit coloration, providing molecular targets for breeding strategies aimed at enhancing phytochemical content (An et al., 2022). Proteomics-based approaches have also contributed substantially to understanding metabolic regulation in medicinal plants. Proteomic

analyses of *Dendrobium huoshanense* have identified crotonylated proteins associated with photosynthesis, the Calvin cycle, and metabolic processes related to alkaloid and polysaccharide biosynthesis, highlighting the importance of post-translational modifications in regulating secondary metabolism. Such findings illustrate how proteome-level information complements genomic and transcriptomic data by capturing regulatory mechanisms that operate beyond transcriptional control. The widespread adoption of multi-omics strategies has stimulated the development of comprehensive databases dedicated to medicinal plants. These resources compile genomic, transcriptomic, proteomic, metabolomic, and phytochemical information either for individual species or across multiple taxa, thereby providing integrated platforms for data mining and hypothesis generation. Among these, the Ginseng Genome Database represents one of the earliest and most comprehensive examples, distinguished by the accuracy and completeness of its whole-genome annotations. Similarly, the Global Pharmacopoeia Genome Database (GPGD) aggregates organelle genomes, whole-genome assemblies, and transcriptomic datasets from hundreds of medicinal plant species, offering an extensive comparative framework. In parallel, specialized databases such as MepmiRDB have expanded the scope of medicinal plant omics by incorporating microRNA data, thereby enabling investigations into post-transcriptional regulation of secondary metabolite accumulation. In addition to omics-focused resources, phytochemical databases—including IMPPAT, NPACT, NuBBEDB, and NANPDB—provide curated information on chemical structures, biological activities, taxonomic distribution, and medicinal usage of plant-derived compounds. Together, these databases support integrative analyses that link gene expression patterns, regulatory networks, and metabolite profiles to pharmacological properties, thereby accelerating natural product–based drug discovery efforts (Zhang et al., 2023).

3. Enhancing secondary metabolite production through the advancement of comparative genomics, genetic mapping, pan-genome, and epigenome research

Recent advances in plant genomics have substantially improved our understanding of the genetic foundations underlying specialized metabolite biosynthesis in medicinal plants. Comparative genomic approaches, in particular, have proven highly effective for identifying lineage-specific gene

expansions, functional diversification, and metabolic innovations associated with pharmacologically relevant compounds. By systematically comparing genomes across taxonomic groups, these analyses reveal both conserved biosynthetic cores and species-specific adaptations that shape metabolite diversity. Comparative genomic investigations in *Panax ginseng* have demonstrated that genes encoding oligopeptide transporters (OPT) contribute to the intracellular transport and accumulation of diverse bioactive compounds, highlighting the role of transport processes in secondary metabolism (Su et al., 2018). Similarly, genome-wide comparisons between *Senna tora* and other Fabaceae members uncovered a pronounced enrichment of genes associated with phenylpropanoid, isoflavone, and terpene biosynthetic pathways, suggesting evolutionary selection for specialized metabolic capacity in this species (Kang et al., 2020). These findings underscore how comparative genomics can uncover metabolic traits that are not apparent from single-genome analyses. The utility of comparative genomics is further illustrated by population-level studies. In *Cannabis sativa*, genome comparisons among high-THC female parental lines, cannabidiol-rich CBDRx (cs10) strains, and balanced CBD/THC Cannbio-2 accessions revealed extensive copy number variations (CNVs) in genes involved in cannabinoid biosynthesis (McKernan et al., 2020). Such structural variations provide a molecular explanation for chemotypic diversity and offer valuable targets for breeding and metabolic engineering. Comparable approaches applied to other medicinal plants, including, *Andrographis paniculata, Zingiber officinale*, *Pistacia chinensis* and *Passiflora edulis*, have enabled the identification of genetic determinants influencing metabolite composition and yield. In parallel with comparative genomics, genetic mapping studies play a critical role in linking phenotypic traits to underlying genomic regions. The construction of genetic linkage maps facilitates the identification of loci associated with bioactive compound production and supports marker-assisted selection strategies. However, the genetic complexity of many medicinal plants—including large genome sizes, extended growth cycles, high heterozygosity, and limited domestication history—poses significant challenges for classical mapping approaches. Despite these constraints, linkage maps have been successfully generated using molecular markers such as, restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP), simple sequence repeats (SSR), expressed sequence tag–derived SSRs (EST-SSR) and random amplified polymorphic DNA (RAPD). These strategies have been applied in a range of species,

including, *Cynara scolymus*, *Passiflora edulis, Silene vulgaris*, and *Trifolium pratense*, providing foundational genetic resources for metabolite-focused research. The transition toward high-density marker systems based on single nucleotide polymorphisms (SNPs) and insertion–deletion variants (Indels) has further improved mapping resolution, enabling the development of saturated and highly informative linkage maps.Beyond single-reference genomes, pan-genome analyses have emerged as a powerful framework for capturing intra-species genomic diversity. By integrating core and accessory genomic regions, pan-genome studies reveal genetic variation that contributes to environmental adaptation and phenotypic plasticity. For example, pan-genomic investigations in *Cajanus cajan* demonstrated that habitat-specific and annual environmental factors significantly influence yield-related traits and metabolite profiles (Zhao et al., 2020). These findings highlight the importance of considering population-level diversity when evaluating medicinal plant traits. Epigenomic research adds a regulatory dimension by elucidating how heritable yet reversible modifications influence gene expression without altering DNA sequence. In medicinal plants, epigenetic mechanisms such as DNA methylation have been linked to tissue-specific metabolite accumulation and environmental responsiveness. Developmental methylome analyses in *Catharanthus roseus* revealed coordinated patterns of DNA methylation associated with tissue-dependent regulation of metabolic pathways (Dugé de Bernonville et al., 2020). Similarly, studies in *Panax quinquefolius* demonstrated that low-temperature environments modulate ginsenoside biosynthesis through dynamic cycles of DNA methylation and demethylation, indicating a reversible epigenetic response to climatic conditions (Hao et al., 2020). Collectively, the integration of comparative genomics, genetic mapping, pan-genome analysis, and epigenomic profiling provides a multidimensional framework for dissecting the molecular basis of secondary metabolite production. As genomic resources continue to expand and analytical methods mature, medicinal plants will remain key biological systems for enhancing the sustainable production of pharmacologically valuable compounds.

3.1. Proteomic-Level Investigation of Medicinal Plants for Drug Development Purposes

The pharmacological relevance of medicinal plants is largely attributed to their capacity to synthesize structurally diverse bioactive compounds, whose

production is tightly regulated at multiple molecular levels. Proteomics has emerged as a powerful approach for elucidating the protein-mediated mechanisms underlying secondary metabolite biosynthesis, accumulation, and regulation in medicinal plant systems. By enabling the large-scale identification and quantification of proteins, proteomic analyses provide direct insights into enzymatic activities, regulatory pathways, and post-translational events that cannot be inferred solely from genomic or transcriptomic data. Proteomic research in medicinal plants generally converges around several interrelated objectives that collectively contribute to drug discovery efforts. These include the identification of enzymes involved in metabolite biosynthetic pathways, the characterization of regulatory proteins controlling metabolic flux, and the discovery of novel proteins or peptides with intrinsic pharmacological activity. Rather than operating independently, these research directions are highly interconnected and often addressed simultaneously within integrative experimental designs. Comparative proteomic studies have proven particularly informative for understanding how environmental and cultivation conditions influence metabolite production. For example, global proteome and phosphoproteome analyses performed on *Dendrobium huoshanense* cultivated under greenhouse conditions versus under-forest stone planting systems revealed substantial differences in protein abundance and phosphorylation status. The under-forest cultivation system was associated with enhanced accumulation of polysaccharides and alkaloids, and site-specific phosphorylation changes in key enzymes suggested a regulatory link between protein modification and metabolite biosynthesis (Wu et al., 2022). Such findings illustrate how proteomics can uncover condition-dependent regulatory mechanisms that directly impact pharmacologically relevant traits.Beyond environmental comparisons, proteomic profiling also facilitates the identification of metabolic bottlenecks and pathway-level coordination. By quantifying protein abundance across tissues or developmental stages, researchers can infer which enzymatic steps exert control over metabolite accumulation. This information is particularly valuable for prioritizing candidate genes for metabolic engineering or for optimizing cultivation strategies aimed at enhancing yield.In addition to pathway-oriented investigations, proteomics has been instrumental in the discovery of novel bioactive proteins and peptides from medicinal plants. Advances in mass spectrometry–based peptidomics have enabled the direct isolation and characterization of small peptides with antimicrobial, antiviral, or cytotoxic properties. For instance, peptidomic

analyses of the aerial tissues of *Amaranthus tricolor* led to the identification of several previously unreported antimicrobial peptides belonging to lipid transfer proteins, snakins, and defensins (Moyer et al., 2021). Similarly, proteomic characterization of peptides isolated from *Acacia catechu* revealed two novel candidates exhibiting strong antiviral activity against dengue virus strains, highlighting the therapeutic potential of plant-derived peptides (Panya et al., 2019).Proteomics also plays a critical role in elucidating the regulation of secondary metabolite biosynthesis at the enzymatic level. Among specialized metabolites, terpenoids represent a major class of pharmaceutical interest, alongside alkaloids and phenolic compounds. Terpenoid biosynthesis relies on two conserved metabolic routes that generate the universal precursor isopentenyl diphosphate (IPP): the cytosolic mevalonate (MVA) pathway and the plastid-localized methylerythritol phosphate (MEP) pathway. The coordinated regulation of these pathways determines the production of diverse terpenoid subclasses, including mono-, sesqui-, di-, tri-, and meroterpenoids.Proteomic analyses have provided detailed insights into tissue-specific regulation of terpenoid biosynthesis. Comparative proteome profiling of rhizomes and leaves of *Anemone flaccida* demonstrated that proteins associated with triterpenoid saponin biosynthesis were predominantly upregulated in rhizome tissues, consistent with metabolite accumulation patterns observed at the chemical level (Zhan et al., 2016). Similarly, label-free quantitative proteomic studies conducted across multiple organs of *Panax ginseng* identified a subset of proteins directly involved in ginsenoside biosynthetic pathways, including enzymes from the MVA and MEP pathways, UDP-glycosyltransferases, and oxidoreductases (Van Nguyen et al., 2021). These studies underscore the value of proteomics for linking protein expression dynamics to tissue-specific metabolite production. Collectively, proteomic investigations provide an indispensable layer of information for drug discovery–oriented research in medicinal plants. By integrating protein-level data with genomic, transcriptomic, and metabolomic profiles, proteomics contributes to a systems-level understanding of metabolic regulation and facilitates the rational exploitation of plant-derived compounds for pharmaceutical applications.

3.2. Medicinal Plant Metabolomic Profiles and Primary and Secondary Metabolites' Pharmacological Potential

Metabolomic analysis occupies a central position in pharmacological research on medicinal plants, as it enables the comprehensive characterization of low-molecular-weight compounds that directly determine therapeutic efficacy. Unlike genomics or proteomics, which describe biological potential and regulatory capacity, metabolomics captures the final biochemical output of cellular processes. Consequently, metabolite profiles provide an integrated snapshot of physiological status and are particularly informative for evaluating the quality, safety, and bioactivity of plant-derived medicinal products. Biotechnological strategies aimed at enhancing secondary metabolite production have demonstrated that plant cell, tissue, and organ cultures can serve as efficient platforms for the controlled synthesis of pharmacologically valuable compounds. To date, more than fifty commercial products derived from plant cell cultures have been developed, underscoring the feasibility of metabolite-focused production systems. Tissue and organ cultures are especially advantageous because they reduce environmental variability and allow metabolite accumulation to be optimized under defined growth conditions, thereby improving consistency and yield (Barut et al., 2022). Metabolomic investigations also play a critical role in assessing the qualitative and quantitative variability of medicinal plant materials. Although extraction methods influence metabolite recovery, differences in therapeutic quality are primarily determined by intrinsic metabolite composition rather than processing alone. Medicinal plants exhibit pronounced tissue- and organ-specific metabolite distribution patterns. This spatial heterogeneity reflects the specialized physiological functions of plant organs and has direct implications for medicinal usage (Rai et al., 2021). Numerous studies have demonstrated that biologically active metabolites frequently accumulate in specific tissues. For example, artemisinin biosynthesis in *Artemisia annua* predominantly occurs in leaf tissues, whereas gingerols are mainly stored in the rhizomes of *Zingiber officinale*. Similarly, taxoid compounds responsible for anticancer activity are concentrated in the bark of *Taxus wallichiana*, while isoquinoline alkaloids accumulate in the rhizomes of *Coptis chinensis*. Such tissue-specific localization patterns emphasize the importance of targeted sampling strategies in metabolomic studies.Advanced analytical platforms, including gas chromatography–mass spectrometry (GC–MS), liquid chromatography–tandem mass spectrometry (LC–MS/MS), and nuclear

magnetic resonance (NMR) spectroscopy, have significantly expanded the scope of metabolomic profiling. These technologies enable high-resolution detection, structural elucidation, and quantification of diverse metabolite classes. Metabolomic comparisons across species, tissues, or geographical origins have proven particularly valuable for identifying compounds with pharmacological relevance. For instance, comparative metabolite profiling of roots, stems, and leaves of *Panax notoginseng* collected from distinct regions revealed that saponin composition was conserved between roots and stems but differed markedly in leaf tissues, suggesting alternative plant parts as potential pharmaceutical resources (Gao et al., 2022). In addition to compound identification, metabolomic data contribute to drug discovery by enabling the prioritization of metabolites with therapeutic potential. In a broad metabolomic screening of medicinal plants, specific hydroxylated fatty acids were identified as promising candidates for antiviral drug development (More et al., 2022). Furthermore, metabolome-based analyses of neuroactive medicinal plants—including *Hypericum perforatum*, *Passiflora incarnata*, *Valeriana officinalis*, and *Melissa officinalis*—revealed positive correlations between primary metabolites of the tricarboxylic acid cycle, secondary metabolites such as flavonoids and terpenoids, and the expression of brain-derived neurotrophic factor (BDNF). These findings highlight the relevance of metabolomics for linking chemical composition to neuroprotective and neuropharmacological effects (Gonulalan et al., 2020). Overall, metabolomic profiling provides a critical experimental bridge between plant biochemistry and pharmacological application. When integrated with genomic, transcriptomic, and proteomic datasets, metabolomics enables a systems-level understanding of how metabolic networks generate therapeutically relevant compounds, thereby supporting the rational development of plant-derived drugs.

3.3. Comprehensive Omics Study of Medicinal Plants Specialized Metabolism

Plants synthesize an extraordinary diversity of specialized metabolites that function as signalling molecules, pigments, defense compounds, and bioactive agents with pharmacological relevance. These metabolites form the chemical basis of many therapeutic effects and represent a major evolutionary strategy that enables plants to adapt to fluctuating environmental conditions and biotic pressures. The remarkable diversity of plant specialized metabolism is the

outcome of long-term evolutionary processes, and current estimates suggest that several hundred thousand distinct metabolites are produced across the plant kingdom (Afendi et al., 2012; Ra, 2003). Historically, plants rich in biologically active metabolites have been integral to traditional medical systems and have played a foundational role in the development of modern pharmaceuticals. Numerous contemporary drugs are either directly derived from plant metabolites or represent semi-synthetic modifications of naturally occurring compounds (Butler, 2004; Kinghorn et al., 2011; Pan et al., 2013). Medical traditions such as Ayurveda, Unani, Siddha, and traditional Chinese and Japanese medicine have guided the discovery of many bioactive molecules by systematically documenting the therapeutic use of medicinal plants (Atanasov et al., 2015; McChesney, 2002). Despite their shared origin from relatively simple biochemical precursors, specialized metabolites often exhibit highly complex chemical architectures. These structural features arise from multistep biosynthetic pathways that are shaped by natural selection to optimize ecological function. In contrast to microorganisms—where genes encoding entire biosynthetic pathways are frequently organized into compact clusters—plant biosynthetic genes are typically dispersed throughout the genome. This genomic organization, combined with large genome sizes, genetic redundancy, cellular compartmentalization, and multi-layered regulatory mechanisms, poses significant challenges for elucidating plant metabolic pathways (Heinig et al., 2013; Sweetlove & Fernie, 2013). Integrated omics approaches provide an effective solution to these challenges by enabling the simultaneous analysis of multiple molecular layers. Genomics and transcriptomics identify candidate genes and expression patterns associated with metabolite accumulation, while proteomics reveals enzyme abundance, activity, and post-translational regulation. Metabolomics captures the chemical end products of these processes, thereby linking molecular regulation to observable metabolic phenotypes. When analyzed independently, each omics layer offers a partial view of the system; however, their integration enables a more accurate and comprehensive reconstruction of metabolic networks. Relationship-based analytical strategies, which examine correlations and dependencies among transcripts, proteins, and metabolites, have proven particularly valuable for pathway discovery in plants. By integrating quantitative datasets across omics layers, researchers can reduce false-positive and false-negative associations and strengthen causal inference (Deshmukh et al., 2014). Such integrative analyses facilitate the identification of key regulatory nodes, metabolic branch points, and rate-

limiting enzymatic steps that govern flux through specialized metabolic pathways (Steuer, 2007; Sweetlove et al., 2008).The integration of multi-omics data also supports the development of predictive metabolic models. These models mathematically represent biological components and their interactions, allowing system behavior to be simulated under defined genetic or environmental conditions. In medicinal plant research, metabolic modeling has emerged as a powerful tool for predicting shifts in metabolite production and for prioritizing engineering targets aimed at enhancing the yield of high-value compounds (Rai & Saito, 2016). Recent technological advances have significantly lowered the cost and increased the resolution of high-throughput omics data generation. When combined with robust bioinformatics pipelines, these developments have extended systems-level plant biology beyond classical model species to include non-model medicinal plants characterized by unique and valuable metabolite profiles (Muranaka & Saito, 2013; Wurtzel & Kutchan, 2016). Understanding the biosynthetic logic and regulatory architecture of pharmacologically important metabolites is therefore essential for implementing synthetic biology, tissue engineering, and metabolic engineering strategies that ensure the sustainable supply of plant-derived therapeutic agents (Rai et al., 2017).

3.4. Approaches Used in the Identification of Metabolites with Pharmacological Value

The identification and structural characterization of metabolites with therapeutic potential rely on advanced analytical methodologies capable of resolving complex chemical mixtures. Among the available techniques, nuclear magnetic resonance (NMR) spectroscopy and chromatography-based mass spectrometry platforms—namely gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS)—are the most widely employed tools for comprehensive metabolite profiling. These approaches enable both qualitative and quantitative assessment of low-molecular-weight compounds and provide critical information regarding chemical structure, abundance, and biological relevance. Chromatography–mass spectrometry–based strategies have proven particularly effective for comparing metabolite distributions across different plant organs, developmental stages, and geographical origins. Such comparative analyses allow researchers to identify tissue-specific or environment-dependent variations in metabolite composition that may

influence pharmacological efficacy. For example, metabolomic profiling of roots, stems, and leaves of *Panax notoginseng* collected from distinct cultivation regions revealed that while saponin profiles were largely conserved between roots and stems, leaf tissues displayed markedly different metabolite compositions. These findings suggest that plant parts traditionally considered secondary may also represent valuable sources of bioactive compounds (Gao et al., 2022).Beyond organ-specific comparisons, metabolomic screening has facilitated the discovery of novel compounds with potential therapeutic applications. In a broad survey of medicinal plants using MS-based metabolomics, specific hydroxylated fatty acids were identified as promising candidates for antiviral drug development, illustrating how untargeted profiling can uncover previously unrecognized bioactive metabolites (More et al., 2022). Such approaches are particularly advantageous in early-stage drug discovery, where unbiased detection strategies increase the likelihood of identifying structurally diverse lead compounds. Metabolomic methodologies have also been applied to investigate the biochemical basis of neuropharmacological activity in medicinal plants. Integrated metabolite profiling of neuroactive species, including *Hypericum perforatum*, *Passiflora incarnata*, *Valeriana officinalis*, and *Melissa officinalis*, demonstrated that both primary metabolites associated with central carbon metabolism and secondary metabolites such as flavonoids and terpenoids contribute to neuroprotective effects. Notably, correlations were observed between specific metabolite classes and the expression of brain-derived neurotrophic factor (BDNF), suggesting a mechanistic link between plant metabolite composition and neurological outcomes (Gonulalan et al., 2020). In parallel, NMR spectroscopy provides complementary advantages by enabling non-destructive analysis and absolute structural elucidation without the need for prior separation in certain applications. Although NMR is generally less sensitive than MS-based methods, its high reproducibility and quantitative accuracy make it particularly valuable for confirming metabolite identity and for standardizing herbal preparations. The combined application of NMR and MS-based platforms therefore represents a robust analytical framework for the reliable identification of pharmacologically relevant metabolites.

Overall, the integration of advanced analytical techniques with -omic workflows has substantially expanded the capacity to identify, prioritize, and characterize bioactive compounds from medicinal plants. When coupled with upstream omics data and bioinformatics-driven interpretation, these

approaches provide a systematic foundation for translating plant metabolite diversity into viable pharmaceutical candidates.

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Chapter 7

BIOLOGICAL PROPERTIES OF ENDEMIC MEDICINAL PLANTS FROM TÜRKİYE

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1. INTRODUCTION

Türkiye represents one of the most floristically diverse regions in the temperate zone due to its unique geological history, distinct topographic heterogeneity, and location at the intersection of numerous climate regimes. Anatolia, situated at the crossroads of Europe and Asia, has not only served as a biogeographic bridge but also provided a long-term refuge during Quaternary climate fluctuations, supporting the survival and diversification of species. These historical and environmental dynamics have led to the formation of an extraordinarily rich vascular plant flora characterized by a high level of endemism (Zohary, 1973; Davis, 1971).

In botanical literature, the term endemic refers to taxa whose natural distribution is restricted to a specific geographical area such as a mountain range, island, or country (Erik & Tarıkahya, 2004). Endemism in the Turkish flora is generally classified into paleo-endemic taxa, which represent relict species that previously had wider distribution ranges, and neo-endemic taxa, which emerged as a result of speciation events that occurred relatively recently, particularly during the late Pleistocene and postglacial periods (Davis, 1971; Öner et al., 2021).

Recent floristic syntheses indicate that Türkiye hosts more than 12,000 vascular plant taxa. This figure is comparable to that of the entire European continent (Güner et al., 2012). The fact that approximately 30-34% of these taxa are endemic highlights Türkiye's global importance as a center of plant endemism (Ekim et al., 2000). This extraordinary richness can largely be attributed to the convergence of three major phytogeographic regions (European-Siberian, Mediterranean, and Iranian-Turanian) within a relatively limited land area, characterized by sharp elevation gradients and diverse geological substrates (Davis, 1971; Zohary, 1973).

Apart from their taxonomic and biogeographic importance, Türkiye's endemic plant species are increasingly attracting the attention of the scientific world due to their biological and phytochemical properties. Most of these species live in environmentally challenging habitats such as high mountain steppes, limestone rocky slopes, saline basins, and semi-arid regions. Drought, extreme temperatures, high ultraviolet radiation, and soil stress have triggered the evolution of complex metabolic responses, often manifested in the form of the

synthesis of various secondary metabolites (Gür, 2017). Compounds such as phenolics, flavonoids, terpenoids, and alkaloids are frequently reported in species-rich families such as Lamiaceae, Asteraceae, and Fabaceae, as well as in endemic taxa (Güner et al., 2012).

These secondary metabolites form the biochemical basis for numerous biological activities, including antioxidant, cytotoxic, antimicrobial, and enzyme-inhibiting effects. As a result, plants endemic to Türkiye are increasingly recognized as valuable sources of bioactive compounds for experimental biological and pharmacological research. At the same time, the limited distribution of endemic taxa makes them particularly vulnerable to habitat loss, changes in land use, and climate-related environmental changes, highlighting the need for integrated approaches that link biological research with conservation priorities (Erik & Tarıkahya, 2004; Ekim et al., 2000).

This book chapter aims to summarize the biological characteristics of endemic plant species in Türkiye, emphasizing experimental evidence from contemporary studies. By placing biological activity data within a well-established floristic and biogeographic framework, this chapter aims to contextualize current findings and support future research on Türkiye's endemic plant resources.

2. ENDEMIC PLANT DIVERSITY IN TÜRKİYE

Türkiye's endemic plant diversity is not evenly distributed among taxa, but rather concentrated in specific plant families and ecological niches. Floristic inventories and taxonomic syntheses have consistently shown that a limited number of families account for a disproportionate share of endemic taxa, reflecting both evolutionary history and habitat specialization (Erik & Tarıkahya, 2004; Güner et al., 2012).

Among the most frequently mentioned families in regional and provincial endemic floras are Asteraceae, Lamiaceae, Caryophyllaceae, Fabaceae, and Scrophulariaceae. For example, in a floristic study conducted in the province of Muğla, 414 endemic taxa belonging to 45 families were identified. Asteraceae (51 taxa), Lamiaceae (50 taxa), and Caryophyllaceae (42 taxa) are the families with the richest species, followed by the Scrophulariaceae and Liliaceae families (Yeşilyurt & Akaydın, 2012). These findings are consistent with other provincial studies, such as those conducted in Afyonkarahisar. In

the Afyonkarahisar study, the Asteraceae, Fabaceae, and Lamiaceae families were also among the dominant families in terms of endemic taxa (Yeşilyurt & Akaydın, 2012; Bingöl et al., 2019).

Asteraceae is typically highlighted as one of the largest contributors to Türkiye's endemic flora. Endemic Asteraceae taxa often occur in steppe, rocky limestone, and alpine environments and frequently exhibit localized distributions. Genera such as *Centaurea* and *Verbascum* feature prominently among endemic assemblages, with *Centaurea* species often cited in floristic inventories for their diversity and restricted ranges. Regional surveys confirm that Asteraceae not only dominate in total taxon count but also contribute critically to the pool of narrowly distributed endemics in Mediterranean and transitional zones (Yeşilyurt & Akaydın, 2012; Baser & Buchbauer, 2015; Özbek et al., 2020).

The Lamiaceae family is a floristically extremely rich group in Türkiye. A comprehensive systematic study conducted nationwide identified 48 genera and a total of 782 taxa, approximately 346 (44.2%) of which are endemic; this indicates that Türkiye is a globally significant center of diversity for Lamiaceae. These endemic taxa are particularly adapted to harsh environmental conditions such as dry, rocky, and mountainous habitats, which can trigger fluid metabolic pathways and increase the production of secondary metabolites such as phenolic compounds and terpenoids. These compounds form chemical profiles associated with antioxidant and biological activity in many Lamiaceae species, and therefore Türkiye's endemic Lamiaceae taxa have frequently been selected materials in pharmacological and biological screening studies. However, some existing studies emphasize that most of these species have been addressed at a limited preliminary level with in vitro tests and that more comprehensive biological activity evaluations are needed (Kuşaksız, 2019; Celep & Dirmenci, 2017).

Endemic taxa belonging to families such as Fabaceae and Caryophyllaceae also contribute significantly to regional plant diversity in Türkiye. Endemic species belonging to Fabaceae are frequently found in Irano-Turanian steppe ecosystems and gain a survival advantage in nutrient-poor steppe soils thanks to their nitrogen-fixing abilities, which facilitates their adaptation over large areas (considering general plant geography knowledge and the relationship between Irano-Turanian habitats and plant endemism). Caryophyllaceae taxa,

on the other hand, include numerous species in Türkiye, and members of this family are widespread in saline, gypsum-rich soils and in high-altitude habitats that create isolation, contributing to speciation processes through ecological isolation (the wide taxonomic diversity of Caryophyllaceae in Türkiye and its distribution in different habitats has been highlighted in existing floristic studies) (Özçelik & Muca, 2019; Yeşilyurt & Akaydın, 2012). The phytochemical profiles of Fabaceae species, encompassing flavonoids, isoflavonoids, alkaloids, phenols, and other secondary metabolites, may play important biological roles in both plant defense mechanisms and oxidative stress modulation and cell signaling pathways; these compounds have been identified in many Fabaceae plants. However, experimental biological studies on Türkiye's endemic Fabaceae taxa have been relatively limited, and the potential activities of this group need to be evaluated in greater depth. This situation reveals that Türkiye's floristic diversity still offers extensive research opportunities from both taxonomic and phytochemical and pharmacological perspectives (Yıldırımlı, 2005; Prajapati et al., 2025).

Trends observed in biological studies conducted on Türkiye's endemic taxa reveal that floristic richness does not correspond with ethnobotanical knowledge. Many endemic species are included in studies without traditional use records, so their selection is based more on scientific criteria such as taxonomic relationships, phytochemical expectations, or ecological adaptations; this situation is considered a fundamental feature that distinguishes endemic plant research from classical ethnopharmacological approaches. Although Türkiye is a globally significant center for plant diversity and endemism due to its high mountainous areas and systematically variable soils, there are numerous cases showing that biological characterization is not equally advanced among selected taxa. The biological characteristics of many taxa remain poorly defined, and existing studies are generally conducted within a narrow taxonomic scope and with limited methodological standards; therefore, there is a need for broader taxonomic coverage, methodological standardization, and strengthened integration between floristic, ecological, and biological research (Noroozi et al., 2019). This perspective provides a strategic framework for both the conservation of Türkiye's plant endemism and the full realization of its scientific potential.

3. MAJOR BIOLOGICAL PROPERTIES OF ENDEMIC PLANT SPECIES FROM TÜRKİYE

Before discussing the biological activities of individual endemic plant species in Türkiye, it is important to briefly summarize the general framework within which these species are evaluated. Current studies primarily use *in vitro* screening approaches that focus on extract-based evaluations rather than isolated compounds and employ a limited but repetitive series of bioassays. The most frequently investigated endpoints are antioxidant capacity, cytotoxic and antiproliferative effects, and stress-related biological responses. These tests are important because they are methodologically accessible and relevant to understanding the adaptive biochemical characteristics of endemic taxa shaped by diverse and often challenging ecological conditions. The following subsections summarize the main biological properties reported in the literature, emphasizing experimentally verified results over ethnobotanical claims or speculative pharmacological interpretations.

3.1. Antioxidant Properties

Oxidative stress, which is linked to an imbalance between reactive oxygen species (ROS) production and antioxidant defenses, is implicated in aging and numerous diseases. Consequently, antioxidant screening of plant extracts has become a fundamental component of biological evaluation studies. Phenolic compounds and flavonoids are key contributors to the free radical scavenging and metal chelating activities observed in plant extracts (Albayrak & Kaya, 2019; Kaska, Çiçek & Mammadov, 2025).

The antioxidant potential of several endemic species unique to Türkiye has been investigated. For example, *Haplophyllum myrtifolium* Boiss. (Rutaceae), which is endemic to the flora of Türkiye, has shown significant antioxidant activity in the DPPH and ABTS tests, exhibited a higher radical scavenging capacity compared to n-hexane extracts, and had a significantly high total phenolic content determined spectrophotometrically (Saltan et al., 2025).

Studies on Lamiaceae endemic species also demonstrate antioxidant capacity. The hydroethanolic extracts of *Nepeta italica* subsp. *cadmea* and *Teucrium sandrasicum* exhibited noteworthy DPPH, ABTS, and phosphomolybdenum antioxidant activity, along with HPLC-confirmed phenolic profiles correlating

with measured radical scavenging and reducing effects (Kaska, Çiçek & Mammadov, 2025).

Additionally, studies on *Astragalus argaeus* Boiss. (Fabaceae) have reported measurable antioxidant activities in extracts obtained from both underground and above-ground parts, although relatively weak compared to standards, and identified ferulic acid as the predominant compound (Albayrak & Kaya, 2019).

Beyond single-species studies, multi-taxon investigations have revealed that endemic taxa such as *Helichrysum noeanum*, *Onosma bozakmanii*, and *Sideritis amasiaca* exhibit varying degrees of DPPH radical scavenging and metal chelation properties, often associated with differences in phenolic composition as determined by chromatographic profiling (Emsen, Surmen & Karapinar, 2023).

Collectively, these studies indicate that, although antioxidant potential varies significantly between species and extract types, many Turkish endemic plants possess biologically relevant antioxidant activities that warrant further mechanistic investigation.

3.2. Cytotoxic and Antiproliferative Effects

Assessing cytotoxicity against cancer cell lines is central to early drug discovery. An increasing amount of research is focusing on endemic taxa from Türkiye using *in vitro* models to evaluate their potential antiproliferative effects.

We evaluated the cytotoxic activity of the methanol extracts of *Marrubium rotundifolium* Boiss., an Aegean endemic, across several human cancer cell lines (Caco-2, SH-SY5Y, and PC-3), as well as non-cancer controls. The results showed that the methanol extract inhibited PC-3 cell proliferation, with an IC_{50} value in the low microgram-per-milliliter range. This indicates a potential selective cytotoxic effect *in vitro* (Kose at al., 2023).

For *Astragalus argaeus* Boiss., extracts from both the aerial and underground parts showed weak cytotoxic responses against MCF-7 breast cancer cells in MTT assays, suggesting limited antiproliferative effects under the tested conditions Albayrak & Kaya, 2019).

Other studies have examined phenolic-rich extracts of endemic species, such as *Helichrysum noeanum*, *Onosma bozakmanii*, and *Sideritis amasiaca*. These studies have shown that cytotoxic outcomes can vary according to phenotype and solvent fraction. This finding reinforces the idea that bioactivity depends on both the composition of the extract and the assay system (Emsen, Surmen & Karapinar, 2023).

In summary, current cytotoxicity data from endemic Turkish flora demonstrate modest, species-dependent antiproliferative potential. While some extracts show promising selectivity, further standardized testing across broader tumor panels and mechanistic endpoints is required.

3.3. Protective and Stress-Related Effects

Endemic species often thrive under harsh ecological conditions, and their ability to withstand stress can be correlated with their bioactive secondary metabolite profiles. While fewer studies have directly assessed protective biological responses beyond antioxidant or cytotoxic assays, the available research suggests that endemic Turkish plants may beneficially engage cellular stress mechanisms.

Studies on *Haplophyllum myrtifolium* reveal antioxidant and antimicrobial properties that suggest broader stress-related biochemical capabilities. The phenolic content and IC_{50} values in common radical scavenging tests are consistent with cellular protection paradigms (Saltan et al., 2025).

Multi-species comparisons show that *Helichrysum noeanum* and related taxa with high phenolic diversity exhibit significant radical scavenging activity, widely interpreted as indicative of protective effects against oxidative stress in biological systems (Emsen, Surmen & Karapinar, 2023).

Studies focusing on total phenolic content, flavonoid profiling, and metal chelation capacity further reinforce the concept that Fabaceae endemics, such as Astragalus davisii, accumulate stress-responsive metabolites with plausible protective roles, even if direct cellular protective assays are less frequently reported (Kartal & Yaren, 2024).

An overview of selected endemic plant species from Türkiye, together with their reported biological activities and experimental assays, is summarized in Table 1.

Table 1. Selected Biological Activities of Endemic Plant Species from Türkiye Reported in Recent Literature

Endemic species	**Family**	**Biological activity / assay**	**Key findings**	**Reference**
Haplophyllum myrtifolium Boiss.	Rutaceae	Antioxidant activity (DPPH, ABTS)	Methanol extract showed higher antioxidant activity (IC_{50}: 0.120 mg/mL) than n-hexane; high total phenolic content	Saltan et al., 2025
Astragalus argaeus Boiss.	Fabaceae	Antioxidant (DPPH, FRAP, CUPRAC) & cytotoxic (MTT, MCF-7)	Both extracts showed weak antioxidant and weak cytotoxic effects on MCF-7 cells	Albayrak & Kaya, 2019
Nepeta cadmea Boiss.	Lamiaceae	Antioxidant (DPPH, ABTS, metal chelation), cytotoxic	Water extract had strong radical scavenging; all extracts showed cytotoxic activity in brine shrink test	Kaska et al., 2018
Heliotropium samolifolium subsp. *erzurumicum*	Boraginaceae	Antioxidant (DPPH, total phenolics), DNA damage effects	Root extracts showed higher antioxidant and DNA protective effects	Sağlam & Kandemir, 2020
Astragalus leporinus var. *hirsutus* & related taxa	Fabaceae	Antioxidant, cytotoxic, antimicrobial	LC-MS profiling demonstrated antioxidant and antimicrobial activities	Haşimi et al., 2017

4. FACTORS INFLUENCING BIOLOGICAL ACTIVITY

The biological activity reported for plant extracts is not an intrinsic, fixed characteristic of a species, but rather the result of multiple interacting biological and methodological factors. The plant organ selected for analysis, the extraction strategy employed, and intra- and interspecific variability are among the factors that play a decisive role in shaping the experimental outcomes reported in the literature (Gobbo-Neto & Lopes, 2007; Wink, 2015; Azmir et al., 2013).

The accumulation of secondary metabolites by different plant organs occurs in distinct qualitative and quantitative patterns, reflecting their ecological and physiological functions. Leaves frequently serve as primary sites for phenolic compounds and flavonoids, which play a crucial role in photoprotection and oxidative stress defense. In contrast, roots and rhizomes have been observed to be enriched in alkaloids, terpenoids, and other defense-related metabolites. These metabolites are associated with soil-borne stressors and herbivory (Xu & Wang, 2024). Consequently, extracts derived from the aerial and underground parts of the same species frequently exhibit divergent biological profiles, even when evaluated using identical assay systems. This organ-dependent metabolite distribution has been reported across diverse plant taxa and is considered a fundamental determinant of extract bioactivity (Krzemińska et al., 2020; Jiang et al., 2021).

The extraction methodology constitutes another critical variable influencing the observed biological activity of plant materials. The polarity of the solvent directly impacts the spectrum of compounds extracted, thereby influencing both antioxidant and cytotoxic outcomes. Polar solvents, including methanol, ethanol, and aqueous mixtures, have been shown to be more effective in extracting phenolic acids and flavonoids, which are known to exhibit radical scavenging and reducing activities. Conversely, non-polar solvents are known to facilitate the isolation of lipophilic constituents, including certain terpenoids and fatty acid derivatives, which may contribute differently to biological responses (Sun et al., 2025). As evidenced by numerous comparative studies, the selection of solvent can exert a substantial influence on the magnitude of activity as well as the apparent selectivity of extracts in cell-based assays (Lee et al., 2023). Consequently, observed discrepancies in reported biological effects among studies may frequently be attributable to

methodological variations rather than to genuine biological inconsistency (Dai & Mumper, 2010; Azmir et al., 2013).

Beyond methodological concerns, biological variability at both interspecific and intraspecific levels further complicates the interpretation of activity data. It is noteworthy that closely related species within the same genus may exhibit significantly divergent phytochemical profiles and biological activities, despite the presence of shared taxonomic characteristics. Furthermore, populations of the same species growing under different ecological conditions may exhibit differences in metabolite composition due to environmental pressures such as altitude, soil composition, climate, and biotic stress. These environmentally driven differences can translate into substantial variation in measured biological activity, even when plant material is processed under standardized laboratory conditions (Sarker & Nahar, 2012; Wink, 2015).

When considered collectively, these factors underscore the imperative for meticulous interpretation when comparing biological activity data across studies. The potential disparities in antioxidant or cytotoxic capacity may not only stem from species-specific characteristics but also from factors such as organ selection, extraction methodology, and ecological variations. It is imperative to acknowledge these influences to contextualize experimental findings and to avoid overgeneralizing biological activity based solely on isolated screening results (Itam et al., 2021; Ziemlewska et al., 2024).

5. LIMITATIONS AND FUTURE PERSPECTIVES

Türkiye's rich endemic plant diversity faces serious threats from increasing anthropogenic pressures and global environmental changes. Endemic plant species are generally restricted in distribution and therefore respond more sensitively than other species to processes such as habitat loss and degradation. According to the International Union for Conservation of Nature (IUCN) criteria, hundreds of endemic plants in Türkiye fall into risk categories such as Critically Endangered (CR), Endangered (EN), and Vulnerable (VU); this is known from both field monitoring studies and national databases (Türe & Böcük, 2010; Keser et al., 2020).

Human-induced habitat changes, agricultural expansion, overgrazing, mining, tourism infrastructure, and urbanization directly destroy the habitats of these narrowly distributed plant communities. In the analysis by Tür and Böcük

(2010), it was noted that a large proportion of endemic plants are located within critically shrinking areas according to IUCN criteria, and only a small proportion of these species are found in existing protected areas; this situation indicates that the current protection system lacks the capacity to cover taxa at risk. High-altitude endemic plant communities are particularly sensitive to the effects of climate change; rising temperatures and changing precipitation regimes can reduce the habitats of alpine and subalpine species, leading to "mountain top squeeze" scenarios, an effect that has also been documented in the literature in the context of other Mediterranean basin endemic floras (Noroozi et al., 2019).

The IUCN Red List and national assessments have revealed that a considerable number of endemic species in Türkiye fall into the Critically Endangered (CR), Endangered (EN), and Vulnerable (VU) categories. For instance, land degradation, grazing, and agricultural practices have been identified as the primary drivers of threat to species belonging to economically and ecologically significant families, such as Fabaceae. These assessments facilitate the creation of comprehensive biodiversity maps and establish the foundation for developing species action plans, particularly for taxa that are at risk of extinction (Kuşaksız., 2019; Yılmaz & İdman, 2024).

The existing literature has explored two primary axes of discussion regarding conservation strategies: *in situ* and *ex situ* approaches. *In situ* conservation strategies prioritize the preservation of species within their natural habitats. In this context, protected areas such as national parks, nature reserves, and Important Plant Areas (IPAs) have been designated, and management plans have been proposed for habitats deemed at risk (Sangeetha et al., 2019; Noroozi et al., 2019). However, it is emphasized that the current network of protected areas does not sufficiently cover narrowly distributed taxa, and that smaller-scale conservation units are needed, especially for mountainous and microhabitat-level endemics. *Ex situ* conservation, on the other hand, involves the storage of genetic diversity as a precautionary measure in botanical gardens and seed banks. This approach guarantees the preservation of genetic material for future generations, particularly for taxa that are at high risk (Sangeetha et al., 2019).

In considering future prospects, the prevailing scholarly consensus underscores the integration of conservation strategies founded upon scientific

data with the incorporation of climate change projections, species distribution models, and habitat degradation scenarios. In order to protect endemic species in Türkiye, it is necessary to develop not only species-level action plans, but also ecosystem and landscape-scale management strategies. The sustainability of narrowly distributed taxa is closely linked to habitat integrity (Türe & Böcük, 2010; Kindlmann et al., 2025).

6. CONCLUSION

Türkiye's remarkable plant biodiversity is attributable to its intricate geological history, pronounced environmental heterogeneity, and protracted evolutionary processes. These elements collectively position Türkiye as a pivotal center of plant endemism within the regional context. Concurrently, the restricted distributions and habitat specificity of many endemic taxa render them particularly vulnerable to anthropogenic pressures and ongoing environmental change. The effective conservation of this biological heritage is contingent upon the expansion and effective management of protected areas, the broader implementation of species-specific conservation action plans, and the inclusion of local communities in conservation efforts through education and awareness. Concurrently, mounting interdisciplinary research endeavors aimed at elucidating the ecological and pharmacological potential of endemic flora may contribute to both biodiversity conservation and the sustainable utilization of biological resources. Within this framework, Türkiye's endemic flora should be regarded not only as a conservation priority, but also as a strategic natural resource that necessitates long-term, science-based management.

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Chapter 8

METHAMPHETAMINE-INDUCED NEUROTOXICITY: OXIDATIVE STRESS, NEUROINFLAMMATION, AND CELLULAR DAMAGE MECHANISMS

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1. Introduction

Methamphetamine (METH) is one of the most widely used synthetic psychostimulants worldwide, and its consumption has shown a significant upward trend, especially in East and Southeast Asia, North America, and parts of Europe (UNODC, 2023). Due to its high lipid solubility, methamphetamine rapidly crosses the blood–brain barrier and accumulates in the central nervous system (Cruickshank & Dyer, 2022). Chronic METH exposure leads to serious neuropsychiatric and neurodegenerative effects, including cognitive impairment, emotional dysregulation, psychosis, behavioral abnormalities, and structural brain damage (Morales et al., 2020).

Research over the past decade reveals that methamphetamine neurotoxicity is primarily mediated by oxidative stress, mitochondrial dysfunction, neuroinflammation, glutamate excitotoxicity, and apoptotic neuronal death (Cadet & Bisagno, 2021; Shiraki et al., 2022). These pathological mechanisms resemble those seen in neurodegenerative diseases, particularly Parkinson's disease, where dopaminergic neurons are selectively vulnerable (Russo &Cavallaro, 2021). This chapter provides a comprehensive overview of recent findings (2020–2024) that elucidate the molecular and cellular mechanisms driving methamphetamine-induced neurotoxicity.

2. Pharmacodynamics and Pharmacokinetics of Methamphetamine

Methamphetamine exerts its effects by dramatically increasing monoamine levels—especially dopamine, norepinephrine, and serotonin—in synaptic clefts. This occurs through multiple mechanisms, including inhibition of dopamine transporter (DAT), vesicular monoamine transporter 2 (VMAT2) disruption, monoamine oxidase (MAO) inhibition, and reverse transport of dopamine through DAT (Herman et al., 2023; García-Cabrerizo & García-Fuster, 2020).

2.1 Entry into Neurons and Dopamine Accumulation

Methamphetamine enters presynaptic neuronal terminals primarily through DAT (Banks et al., 2022). Once inside, it inhibits VMAT2, causing dopamine to leak from synaptic vesicles into the cytoplasm (Sharma et al., 2021).

Cytoplasmic dopamine then undergoes auto-oxidation, generating highly toxic reactive oxygen species (ROS).

2.2 Reverse Dopamine Transport

Methamphetamine reverses the direction of dopamine transporters, causing massive dopamine efflux into the synapse (Cho et al., 2021).

2.3 Disruption of Monoamine Metabolism

METH also inhibits MAO activity, reducing dopamine degradation and further contributing to neurotransmitter accumulation (Kwon et al., 2023).

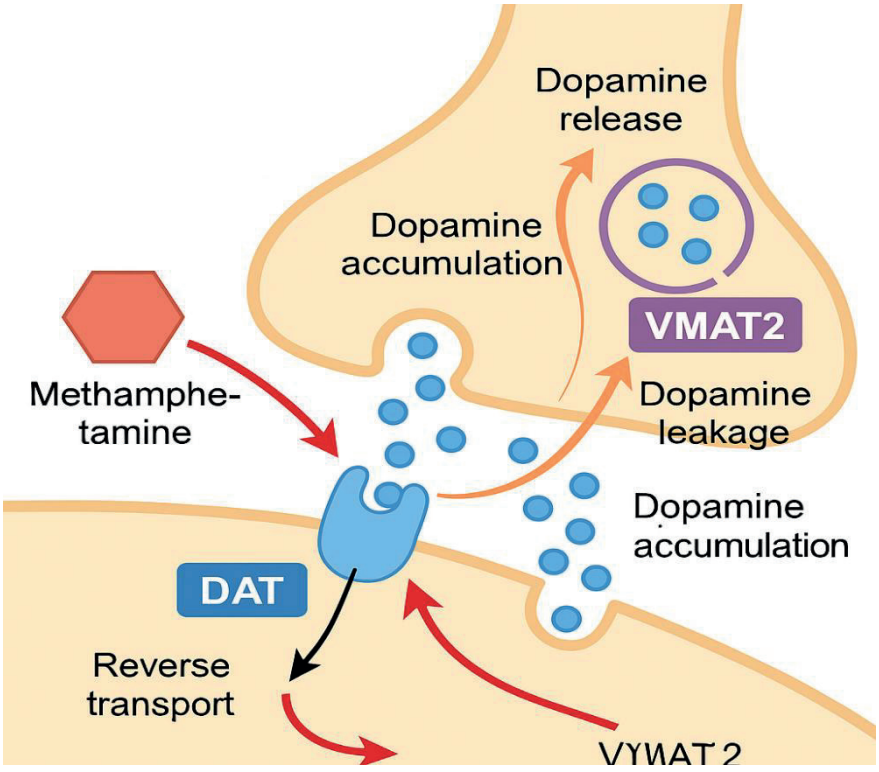

Figure 1. Mechanism of Methamphetamine Action on DAT and VMAT2 Leading to Dopamine Accumulation and Reverse Transport

3. Oxidative Stress Mechanisms

Oxidative stress is a core pathological mechanism in methamphetamine neurotoxicity. Cytosolic dopamine oxidation produces free radicals, including superoxide, hydrogen peroxide, and hydroxyl radicals (Beckmann & Oreland, 2020; Ramsay & Nguyen, 2022). Additionally, methamphetamine disrupts mitochondrial electron transport chain complexes, increasing mitochondrial ROS production (Cao et al., 2023).

3.1 ROS and RNS Production

Methamphetamine increases:

- Superoxide anions ($O_2\bullet-$)
- Hydrogen peroxide (H_2O_2)
- Hydroxyl radicals (•OH)
- Peroxynitrite (ONOO–), driven by nitric oxide elevation (Althobaiti et al., 2022)

3.2 Lipid Peroxidation and Protein Damage

Free radicals attack lipid membranes, leading to lipid peroxidation and membrane instability (Moreira & Silva, 2023). ROS also oxidize proteins and damage DNA, contributing to long-term neuronal dysfunction (Yuan et al., 2021).

3.3 Antioxidant System Collapse

METH decreases important antioxidant enzymes:

- Superoxide dismutase (SOD)
- Catalase (CAT)
- Glutathione (GSH) (Lin & Huang, 2023)

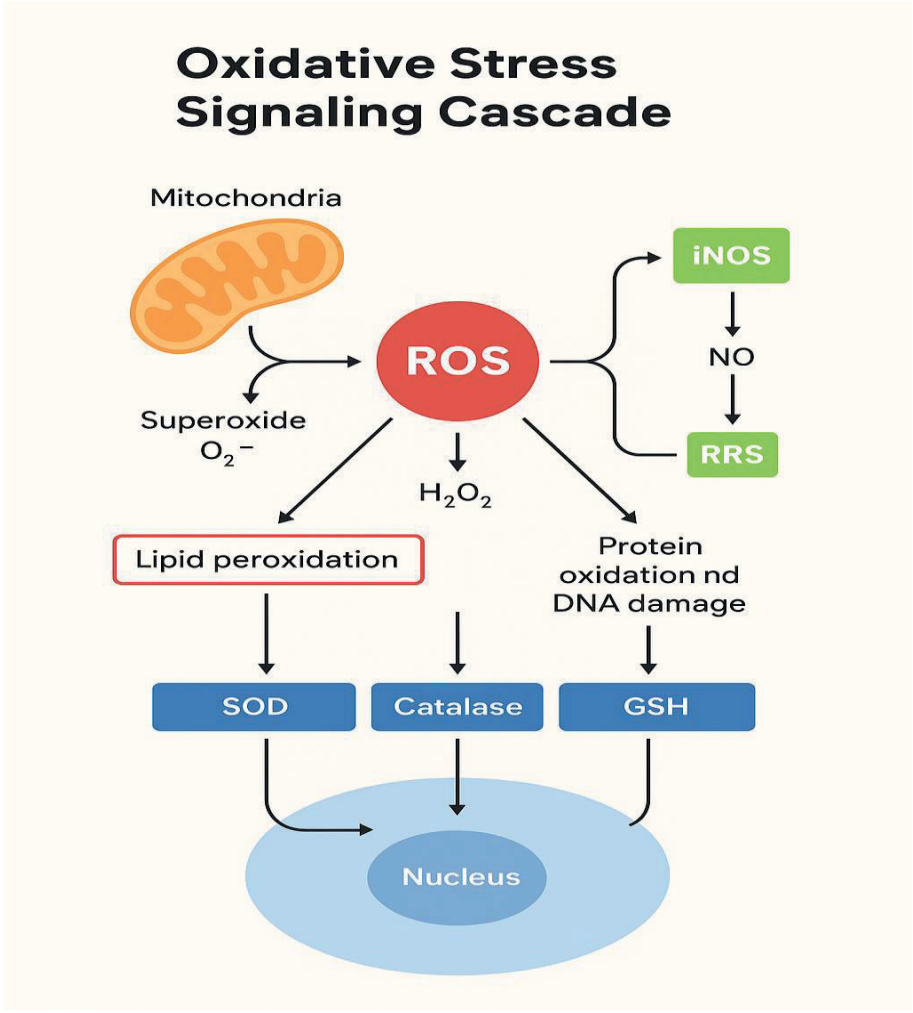

Figure 2. Oxidative Stress Pathway Induced by Methamphetamine: ROS/RNS Formation, Lipid Peroxidation, and Antioxidant System Response

4. Neuroinflammation and Microglial Activation

Neuroinflammation plays a significant role in METH neurotoxicity. Microglia, the resident immune cells of the CNS, become activated after methamphetamine exposure (Martín-Jiménez et al., 2022). Activated microglia release inflammatory cytokines, chemokines, and nitric oxide.

4.1 Pro-inflammatory Cytokines

METH increases:

- IL-1β (Costa et al., 2021)
- IL-6 (Ishikawa et al., 2023)
- TNF-α (Brennan et al., 2022)

4.2 NF-κB and NLRP3 Inflammasome Activation

NF-κB activation is a major step leading to transcription of inflammatory mediators (Kang et al., 2022).

The NLRP3 inflammasome is also activated, contributing to pyroptosis and neurotoxicity (Peng et al., 2021).

4.3 Inducible Nitric Oxide Synthase (iNOS) Activation

iNOS produces large amounts of nitric oxide, which reacts with superoxide to form peroxynitrite, a highly neurotoxic compound (Ribeiro & Alves, 2023).

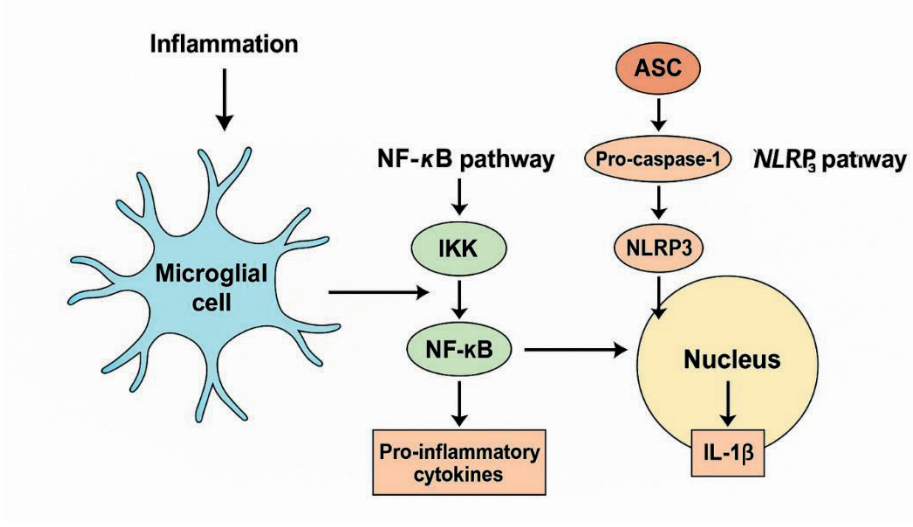

Figure 3. Microglial Activation and NF-κB / NLRP3 Inflammasome Pathways in Methamphetamine-Induced Neuroinflammation

5. Mitochondrial Dysfunction and Apoptosis

Methamphetamine impairs mitochondrial function by disrupting electron transport chain complexes, decreasing ATP production, and increasing mitochondrial membrane permeability (Sun et al., 2023).

5.1 Mitochondrial Membrane Permeability

METH induces mitochondrial swelling, decreased membrane potential, and cytochrome-c release (Zhang et al., 2021).

5.2 Caspase Activation

Released cytochrome-c activates caspase-9 and then caspase-3, leading to apoptosis (Lin & Huang, 2023).

5.3 Bax/Bcl-2 Ratio Shift

METH increases the Bax/Bcl-2 ratio, shifting the balance toward cell death (Hsieh & Chen, 2022).

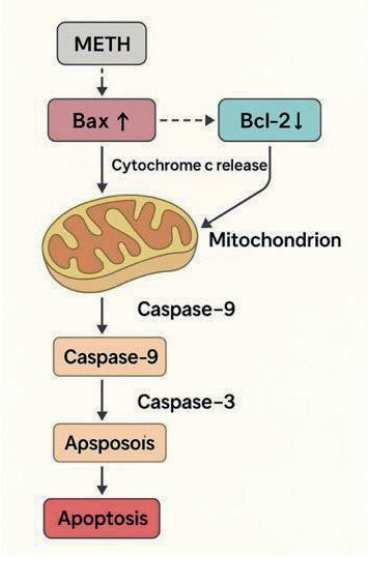

Figure 4. Bax/Bcl-2 Imbalance, Cytochrome-c Release, Caspase Activation, and Apoptosis in Methamphetamine Neurotoxicity

6. Degeneration of the Dopaminergic System

The dopaminergic system is particularly vulnerable to methamphetamine toxicity. METH exposure reduces striatal dopamine, damages dopaminergic terminals, and decreases tyrosine hydroxylase (TH) expression (Shin et al., 2022).

Neuroimaging studies show Parkinson's disease like DAT reductions in chronic METH users (Asanuma et al., 2021; NIDA, 2022).

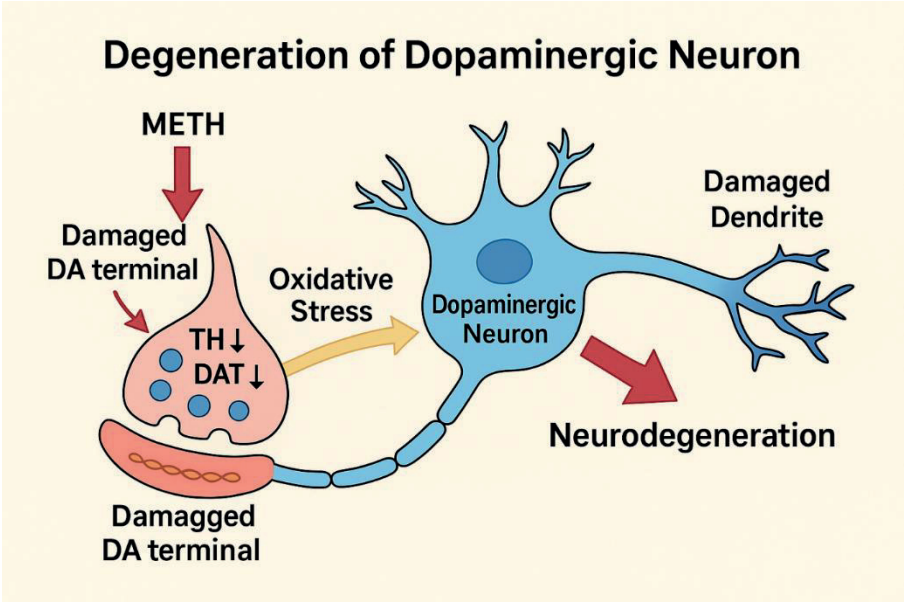

Figure 5. Dopaminergic Neuron Degeneration Induced by Methamphetamine: Loss of TH/DAT, Oxidative Stress, Terminal Damage, and Dendritic Degeneration

7. Behavioral Impairments

Chronic methamphetamine exposure produces severe behavioral and cognitive impairments.

7.1 Cognitive Dysfunction

METH reduces working memory, spatial learning, and executive functions (Chang et al., 2022).

7.2 Affective Disorders

METH induces:

- Anxiety-like behavior (Yuan et al., 2022)
- Depressive-like behavior (Hernández et al., 2023)

7.3 Common Behavioral Tests

- Y-Maze
- Novel Object Recognition
- Morris Water Maze
- Elevated Plus Maze
- Open Field Test

8. Systemic Toxicity: Heart, Liver, Kidney

Methamphetamine toxicity is not limited to the CNS; it affects multiple organs.

8.1 Cardiovascular

- Arrhythmias
- Myocardial ischemia (Rosenthal & Shi, 2022)

8.2 Liver

- Hepatocellular swelling
- ALT/AST elevation (Li et al., 2023)

8.3 Kidney

- Acute kidney injury

- Rhabdomyolysis-induced nephropathy (Liu et al., 2022; Cao et al., 2023)

9. Treatment and Protective Strategies

9.1 Antioxidants

- N-acetylcysteine (Simmons & Miranda, 2021)
- Vitamin E (Moreira & Silva, 2023)

9.2 Anti-inflammatory

- Curcumin (Zhu et al., 2022)
- Omega-3 fatty acids (Becker & Klein, 2021)

9.3 Natural Compounds

- Betanin (Hsieh & Chen, 2022)
- Flavonoids (Thakur & Halder, 2022)

9.4 Experimental Approaches

- Caspase inhibitors
- VMAT2 stabilizers (Sharma et al., 2021)

10. Conclusion

Recent evidence confirms methamphetamine-induced neurotoxicity involves oxidative stress, neuroinflammation, mitochondrial dysfunction, and apoptotic pathways. These mechanisms converge to damage dopaminergic neurons, impair cognition, and disrupt emotional regulation. Understanding these pathways may lead to development of targeted therapies that combine antioxidant, anti-inflammatory, and neuroprotective strategies.

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Chapter 9

REDOX HOMEOSTASIS AND ROS SIGNALING NETWORKS IN PLANTS EXPOSED TO HEAVY METALS

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1. Introduction

Heavy metal contamination of agricultural soils has emerged as a major constraint on plant productivity and global food security, driven largely by rapid industrialization, mining activities, wastewater irrigation and the excessive use of agrochemicals (Gupta et al., 2023; Hasanuzzaman et al., 2020). Metals such as cadmium (Cd), lead (Pb), arsenic (As) and mercury (Hg) are non-essential elements with no recognized physiological roles in plants. In contrast, copper (Cu), zinc (Zn) and iron (Fe) are indispensable micronutrients that readily become toxic when their intracellular concentrations exceed tightly regulated optimal thresholds (Gupta et al., 2023). Irrespective of their essentiality, elevated metal levels commonly perturb cellular redox homeostasis and promote excessive generation of reactive oxygen species (ROS), a defining feature of metal-induced oxidative stress (Hasanuzzaman et al., 2020).

For many years, ROS were viewed predominantly as unavoidable and harmful by-products of aerobic metabolism, responsible for lipid peroxidation, protein oxidation, nucleic acid damage and, ultimately, growth inhibition and cell death (Mittler, 2017; Waszczak et al., 2018). This simplified "ROS as toxic molecules" paradigm, reinforced by early observations of oxidative injury under abiotic stresses including heavy metal exposure, has since been revised. Accumulating evidence now demonstrates that ROS also function as central signaling entities that integrate environmental inputs with developmental programs and stress-response pathways (Liu et al., 2024; Wang et al., 2025). When generated in a spatially and temporally controlled manner and maintained at low to moderate concentrations, specific ROS species participate in finely tuned signaling cascades that regulate root system architecture, stomatal dynamics, reproductive development and adaptive stress responses (Liu et al., 2024; Waszczak et al., 2018).

Under heavy metal stress, ROS occupy a pivotal position at the interface between toxicity and tolerance. Metals interfere with electron transport chains and redox-active enzymes in chloroplasts, mitochondria and peroxisomes and simultaneously stimulate plasma membrane–localized NADPH oxidases, collectively enhancing the production of superoxide anions, hydrogen peroxide, singlet oxygen and hydroxyl radicals (Gupta et al., 2023; Sharma et

al., 2025). Excessive and uncontrolled accumulation of these species disrupts redox equilibrium, accelerates oxidative damage and promotes programmed cell death, particularly in metal-sensitive genotypes or under high-dose exposure scenarios (Sharma et al., 2025). By contrast, moderate and compartmentalized ROS signals activate antioxidant defense systems, metal transporters, chelators and metabolic reprogramming pathways, thereby facilitating acclimation and improved metal tolerance. This functional duality underlies the well-established "double-edged sword" concept of ROS action in plant stress biology (Liu et al., 2024; Mittler, 2017).

Central to this dual role is redox homeostasis, defined as the dynamic balance between ROS generation and scavenging, which is governed by enzymatic and non-enzymatic antioxidant networks and key redox couples such as ascorbate/dehydroascorbate and glutathione (GSH/GSSG) (Foyer & Noctor, 2011; Noctor et al., 2014). Under heavy metal stress, perturbations in these buffering systems modify cellular redox potential and influence the activity of redox-sensitive proteins, including kinases, phosphatases, transcription factors and ion transporters (Foyer & Noctor, 2011; Gupta et al., 2023). Through oxidative post-translational modifications such as S-nitrosylation, S-glutathionylation and disulfide bond formation, ROS and related reactive nitrogen species modulate signaling pathways and gene expression programs that govern metal uptake, sequestration, detoxification and cellular repair processes (Domingos et al., 2015; Kolbert et al., 2019). In this context, redox homeostasis functions not merely as a protective mechanism but as an information-processing hub that integrates metal-derived and developmental signals into context-dependent physiological outputs (Foyer & Noctor, 2011; Liu et al., 2024).

Heavy metal–induced ROS signaling operates within a broader regulatory network that encompasses calcium (Ca^{2+}) dynamics, mitogen-activated protein kinase (MAPK) cascades, phytohormonal signaling and transcriptional regulation. Increases in apoplastic and cytosolic ROS frequently coincide with transient Ca^{2+} signatures, activation of calcium-dependent protein kinases and MAPKs and subsequent modulation of stress-responsive transcription factors, including members of the WRKY, NAC and bZIP families (Cuypers et al., 2016; Niekerk et al., 2024). Concurrently, metal stress reshapes the crosstalk between ROS and phytohormones such as salicylic acid, jasmonic acid, ethylene, abscisic acid and melatonin, which

collectively coordinate defense activation, growth modulation and resource allocation (Hasanuzzaman et al., 2020; Zhang et al., 2023). The balance achieved through these interactions ultimately determines whether ROS signaling supports survival through acclimation or contributes to irreversible cellular damage and death (Liu et al., 2024; Wang et al., 2025).

The aim of this review is to provide an integrated perspective on the mechanisms by which heavy metals disrupt redox balance, stimulate ROS production and engage ROS-dependent signaling networks in plants. The following sections (a) summarize metal uptake, transport and subcellular accumulation processes that predispose cells to enhanced ROS generation, (b) describe the principal cellular sources of ROS and the organization of redox homeostasis under metal stress, (c) examine the signaling functions of distinct ROS species and their integration with Ca^{2+} signaling, MAPK cascades and transcriptional networks and (d) analyze the interplay between ROS and phytohormones in shaping adaptive redox responses and metal tolerance (Cuypers et al., 2016; Gupta et al., 2023). Finally, the review discusses how emerging insights into metal–ROS–redox interactions may be exploited in breeding and biotechnological strategies and how omics-based and redox-proteomic approaches can uncover novel regulatory nodes and molecular targets for improving metal stress resilience (Foyer & Noctor, 2011; Niekerk et al., 2024).

2. Metal Uptake and ROS Generation under Heavy Metal Stress

Heavy metal–induced ROS formation in plants is closely linked to the routes by which metals are taken up, transported and compartmentalized at tissue and cellular scales. Following entry into the plant, metals are redistributed through the vascular system and accumulate in specific subcellular compartments, where they interfere with redox-sensitive metabolic processes and stimulate ROS production. The subsections below summarize the principal mechanisms of metal uptake and cellular accumulation and outline the major intracellular sites of ROS generation under heavy metal stress (Mansoor et al., 2023; Zheng et al., 2025).

2.1. Metal Uptake and Cellular Accumulation

Heavy metals enter plants predominantly through the root system, where uptake proceeds via both apoplastic and symplastic pathways depending on

metal speciation, soil pH and rhizosphere chemistry (Mansoor et al., 2023; Zheng et al., 2025). At the plasma membrane of root epidermal and cortical cells, transport proteins that originally evolved to mediate the acquisition of essential nutrients frequently facilitate the influx of toxic metal ions. These include members of the ZIP, NRAMP, HMA and COPT transporter families (Berni et al., 2019; Ali et al., 2025). For example, Fe and Zn transporters readily translocate Cd^{2+}, while Ca^{2+} channels and K^{+} transport systems may unintentionally permit the entry of other non-essential cations, underscoring an inherent selectivity limitation at the root–soil interface (Mansoor et al., 2023; Zheng et al., 2025).

Once inside the root symplast, metals are transported radially toward the stele and subsequently loaded into the xylem for long-distance translocation. This process often involves complexation with organic ligands, including organic acids, amino acids and nicotianamine, which enhance metal solubility and mobility while buffering free ionic activity (Das & Roychoudhury, 2014; Berni et al., 2019). HMA-type transporters and related efflux systems mediate the export of metal–ligand complexes into xylem vessels, enabling root-to-shoot transport driven by the transpiration stream. In parallel, phloem-based redistribution supplies metals to metabolically active sinks such as developing leaves, meristems and reproductive organs (Das & Roychoudhury, 2014; Zheng et al., 2025).

At the cellular level, plants display pronounced heterogeneity in metal accumulation patterns, reflecting distinct detoxification and sequestration strategies. A primary defense mechanism involves binding of metals to cell wall components within the apoplast, where negatively charged pectins and hemicelluloses immobilize cations and restrict their entry into the cytosol (Das & Roychoudhury, 2014; Berni et al., 2019). Metals that reach the cytosol are rapidly chelated by ligands such as glutathione-derived phytochelatins and cysteine-rich metallothioneins, thereby reducing the pool of free ions and facilitating sequestration into vacuoles via tonoplast-localized transporters, including members of the MTP, CAX and HMA families (Ali et al., 2025; Zheng et al., 2025). Chloroplasts and mitochondria may also accumulate substantial metal loads owing to specific transport systems in their envelopes and the requirement for metal cofactors in photosynthetic and respiratory complexes. However, excessive metal accumulation in these organelles destabilizes electron transport processes and markedly increases their

susceptibility to ROS overproduction under stress conditions (Mansoor et al., 2023; Ali et al., 2025).

Subcellular compartmentalization therefore generates distinct pools of metals with contrasting physiological consequences. Retention within the apoplast or vacuole limits direct interference with redox-sensitive enzymes, whereas accumulation in chloroplasts, mitochondria and peroxisomes substantially increases the likelihood of redox cycling and ROS amplification (Berni et al., 2019; Mansoor et al., 2023). Hyperaccumulator species typically exhibit enhanced expression or activity of uptake transporters, xylem loaders and vacuolar or organellar sequestration systems, enabling them to tolerate exceptionally high metal concentrations while maintaining comparatively controlled ROS levels. In contrast, metal-sensitive species often suffer from inefficient compartmentalization, leading to metal mislocalization and rapid oxidative damage (Ali et al., 2025; Zheng et al., 2025).

2.2. Cellular ROS-Generating Sites under Heavy Metal Stress

Under non-stress conditions, ROS are continuously produced as unavoidable by-products of aerobic metabolism, primarily in chloroplasts, mitochondria and peroxisomes, with additional contributions from the plasma membrane, endoplasmic reticulum and cell wall (Das & Roychoudhury, 2014; Zheng et al., 2025). Exposure to heavy metals perturbs the redox balance of these compartments, enhances electron leakage to molecular oxygen and promotes the formation of superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals (Berni et al., 2019; Mansoor et al., 2023). In chloroplasts, metals interfere with components of the photosynthetic electron transport chain, particularly at photosystems I and II, disrupting electron flow and increasing the probability of oxygen reduction, which results in elevated production of superoxide and singlet oxygen under illuminated conditions (Mansoor et al., 2023; Zheng et al., 2025).

Mitochondria represent another major source of ROS during heavy metal stress. Inhibitory effects or mismetallation of respiratory complexes, especially complexes I and III, promote electron backflow and enhance superoxide formation at multiple sites within the electron transport chain (Mansoor et al., 2023; Zheng et al., 2025). Metabolic coupling between chloroplasts and

mitochondria, including malate and other redox shuttles that transfer reducing equivalents, can further synchronize ROS production in these organelles and intensify oxidative pressure as metal-induced damage accumulates (Zheng et al., 2025). Peroxisomes also contribute substantially to the cellular ROS pool. Metals can modulate the activity of peroxisomal oxidases and catalase, shifting the balance toward hydrogen peroxide accumulation during processes such as photorespiration and fatty acid β-oxidation (Das & Roychoudhury, 2014; Mansoor et al., 2023).

Beyond intracellular organelles, the plasma membrane constitutes a key site of metal-induced ROS production through the activity of NADPH oxidases, also known as respiratory burst oxidase homologs (RBOHs). These enzymes catalyze the transfer of electrons from cytosolic NADPH to apoplastic oxygen, generating superoxide that is rapidly dismutated to hydrogen peroxide and gives rise to a characteristic oxidative burst in the cell wall space (Mansoor et al., 2023; Zheng et al., 2025). Heavy metal exposure frequently activates RBOHs via calcium-dependent and phosphorylation-mediated regulatory mechanisms, thereby linking early metal perception events to ROS-based signaling at the cell surface (Mansoor et al., 2023; Ali et al., 2025). Apoplastic ROS generated by RBOHs not only contribute to local cell wall modification and defense responses but also diffuse into adjacent cells or re-enter the cytosol, where they integrate with intracellular ROS signals originating from chloroplasts, mitochondria and peroxisomes (Das & Roychoudhury, 2014; Berni et al., 2019).

Collectively, heavy metals reshape the spatial and temporal patterns of ROS generation across multiple cellular compartments, producing a complex mosaic of oxidative microenvironments. The relative contribution of chloroplastic, mitochondrial, peroxisomal and apoplastic ROS sources depends on the metal species, its concentration, exposure duration and environmental context, including light conditions and oxygen availability (Mansoor et al., 2023; Zheng et al., 2025). The ability of plants to perceive and interpret these compartment-specific ROS signatures—channeling them toward adaptive signaling or allowing them to escalate into damaging oxidative stress—critically depends on the efficiency of downstream antioxidant systems and redox-regulated signaling networks, which are addressed in the following sections.

3. Redox Homeostasis and Antioxidant Defense under Heavy Metal Stress

Heavy metal exposure profoundly reshapes cellular redox balance in plants by simultaneously enhancing the generation of reactive oxygen species (ROS) and challenging the capacity of antioxidant systems to maintain redox homeostasis (Gupta et al., 2023; Hasanuzzaman et al., 2020). In this section, the concept of redox homeostasis is discussed with particular emphasis on the central role of low-molecular-weight redox couples, followed by an overview of the major enzymatic and non-enzymatic antioxidant components that sustain this balance under heavy metal stress (Foyer & Noctor, 2011; Li et al., 2022). Collectively, these mechanisms determine whether metal-induced ROS remain within a signaling-compatible range or instead overwhelm cellular defenses, leading to oxidative damage and cell death (Bielen et al., 2013; Wang et al., 2023).

3.1. What is Redox Homeostasis?

In plant cells, redox homeostasis refers to the dynamic equilibrium between oxidizing and reducing reactions, primarily governed by the balance between ROS production and the capacity of antioxidant and redox-buffering systems to detoxify these species or channel them into regulated signaling pathways (Foyer & Noctor, 2011). This equilibrium is inherently flexible rather than static, fluctuating within a physiological window that allows ROS to function as signaling molecules without provoking excessive oxidative injury. Heavy metal stress perturbs this balance by stimulating ROS generation in major organelles and at the plasma membrane, thereby shifting cells toward a more oxidizing intracellular environment unless counteracted by adaptive modulation of redox buffers and antioxidant defenses (Gupta et al., 2023).

Low-molecular-weight redox couples, particularly ascorbate/dehydroascorbate (AsA/DHA) and reduced/oxidized glutathione (GSH/GSSG), occupy a central position in redox homeostasis and together constitute a redox hub that links metabolism, development and stress responses (Foyer & Noctor, 2011; Li et al., 2022). Importantly, the ratios of these redox pairs, rather than their absolute pool sizes alone, are key determinants of cellular redox potential across different compartments, including the cytosol, chloroplasts, mitochondria and apoplast (Bielen et al.,

2013). Under heavy metal stress, alterations in the GSH/GSSG and AsA/DHA ratios reflect shifts in ROS load and antioxidant activity and can influence the redox state of proteins through reversible thiol-based modifications, thereby modulating signaling pathways and gene expression programs (Bielen et al., 2013; Gupta et al., 2023).

The ascorbate–glutathione (AsA–GSH) cycle represents a core functional module within this redox network, coupling the detoxification of hydrogen peroxide with the regeneration of AsA and GSH via the coordinated activities of ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase (Foyer & Noctor, 2011; Li et al., 2022). Exposure to heavy metals commonly induces this cycle as part of an adaptive response aimed at stabilizing redox homeostasis. However, under prolonged or severe metal stress, depletion of AsA and GSH pools or a shift toward their oxidized forms may occur, resulting in a more oxidized cellular redox potential (Bielen et al., 2013; Wang et al., 2023). Notably, moderate and compartment-specific changes in redox potential are now recognized as integral elements of ROS-dependent signaling rather than mere indicators of cellular damage, underscoring the dual protective and information-processing roles of redox homeostasis in plant stress responses (Foyer & Noctor, 2011; Wang et al., 2023).

3.2. Antioxidant Defense Systems (Enzymatic and Non-Enzymatic)

To maintain redox homeostasis under fluctuating ROS levels, plants deploy an extensive antioxidant defense network composed of enzymatic and non-enzymatic components distributed across cellular compartments. The principal enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and additional peroxidases and reductases that support the operation of the AsA–GSH cycle (Ansari et al., 2015; Cannea et al., 2025). SOD constitutes the primary line of defense against superoxide radicals by catalyzing their conversion to hydrogen peroxide and molecular oxygen in chloroplasts, mitochondria, peroxisomes and the cytosol (Gupta et al., 2023). The resulting hydrogen peroxide is subsequently removed by CAT, which operates efficiently at relatively high H_2O_2 concentrations in peroxisomes, and by APX, which uses AsA as an electron donor and functions at lower H_2O_2 concentrations in multiple compartments, thereby enabling fine spatial and temporal regulation

of H_2O_2 levels compatible with signaling processes (Cannea et al., 2025; Li et al., 2022). GR plays a critical role in sustaining redox balance by maintaining glutathione in its reduced form using NADPH, thus preserving the GSH/GSSG ratio and supporting continued operation of the AsA–GSH cycle under heavy metal stress (Ansari et al., 2015; Gupta et al., 2023).

Non-enzymatic antioxidants act in concert with these enzymatic systems to buffer ROS and modulate redox signals. Ascorbate and glutathione are the most abundant soluble antioxidants in plant cells, capable of directly scavenging ROS, regenerating other antioxidants and serving as cofactors or substrates in key detoxification reactions (Foyer & Noctor, 2011; Bielen et al., 2013). In addition, secondary metabolites such as phenolic compounds, flavonoids, carotenoids and tocopherols contribute to antioxidant capacity and metal chelation, particularly within the apoplast, vacuole and chloroplast membranes, where they protect structural components and limit oxidative damage (Gupta et al., 2023; Kısa, 2018). Under heavy metal stress, both enzymatic and non-enzymatic components of the antioxidant system are frequently upregulated or reconfigured, and the effectiveness of this coordinated response often correlates with the degree of metal tolerance observed among different genotypes and species (Ansari et al., 2015; Li et al., 2022).

These relationships are summarized in Table 3.1, which presents the principal antioxidant components, their predominant subcellular localization, major functions and documented roles in mitigating oxidative stress under heavy metal exposure.

Table 3.1. Major antioxidants involved in redox homeostasis under heavy metal stress

Antioxidant	*Localization*	*Main function*	*Relationship with heavy metal stress*
SOD (Cu/Zn-, Mn-)	Chloroplast, mitochondria, peroxisome, cytosol	Dismutation of superoxide to H_2O_2 and O_2	Activity typically increases to limit superoxide buildup under metal-induced oxidative stress (Gupta et al., 2023; Cannea et al., 2025).
CAT	Peroxisomes	High-capacity H_2O_2 detoxification	Often induced during prolonged metal exposure to remove excess H_2O_2 (Ansari et al., 2015).
APX (various Isoforms)	Chloroplast stroma/thylakoid, mitochondria, cytosol, peroxisomes	AsA-dependent fine-tuning of H_2O_2 levels	Plays a key role in maintaining signaling-compatible H_2O_2 under metal stress (Cannea et al., 2025; Li et al., 2022).
GR	Chloroplast, mitochondria, cytosol	Regeneration of GSH from GSSG	Supports GSH/GSSG balance and the AsA–GSH cycle in metal-stressed tissues (Ansari et al., 2015).
Ascorbate	Cytosol, chloroplast, mitochondria, apoplast	Direct ROS scavenging; APX substrate and cofactor	Pool size and redox state are markedly affected by excess metals (Bielen et al., 2013).
Glutathione	Cytosol, chloroplast,	Redox buffer; substrate	Involved in both ROS detoxification and metal

	mitochondria, nucleus	for phytochelatin synthesis	chelation/detoxification (Gupta et al., 2023; Li et al., 2022).
Phenolics/ FlavonoIds	Apoplast, vacuole, chloroplast envelope and thylakoids	ROS scavenging; metal chelation; membrane protection	Frequently accumulate under heavy metal stress and contribute to non-enzymatic antioxidant capacity (Gupta et al., 2023; Kısa, 2018).

4. ROS as Signaling Molecules under Heavy Metal Stress

Heavy metal stress not only stimulates the overproduction of reactive oxygen species (ROS) but also alters their spatial and temporal distribution in ways that carry substantial signaling information (Mittler, 2017; Waszczak et al., 2018). In plant cells, the qualitative and quantitative profiles of ROS determine whether these molecules primarily contribute to oxidative damage or instead act as second messengers that modulate stress perception, gene expression and physiological acclimation (Cuypers et al., 2016; Gupta et al., 2023). Building on the concepts introduced in Sections 2 and 3, metal-induced ROS signals are now recognized as highly compartmentalized, species-specific and tightly integrated with broader signaling networks involving calcium, protein kinases and transcription factors (Foyer & Noctor, 2011; Wang et al., 2025).

4.1. Species-Specific ROS Signaling under Heavy Metal Stress

Different ROS species exhibit distinct physicochemical properties that critically shape their signaling potential under heavy metal stress. Superoxide ($O_2\bullet^-$) is a relatively short-lived and poorly diffusible radical, primarily generated at defined hotspots of electron leakage in chloroplasts and mitochondria, whereas hydrogen peroxide (H_2O_2) is more stable, membrane-permeable and capable of moving between compartments via aquaporins (Waszczak et al., 2018; Wang et al., 2025). Singlet oxygen ($^1O_2$), formed mainly at photosystem II under excess excitation pressure, and hydroxyl radicals (•OH), produced through Fenton-type reactions, are extremely reactive with very limited diffusion distances and are therefore generally regarded as damage-promoting rather than classical signaling molecules (Mittler, 2017;

Hasanuzzaman et al., 2020). Under heavy metal exposure, the relative contribution of each ROS species depends on the metal involved, its redox activity and its effects on photosynthetic or respiratory electron transport chains (Gupta et al., 2023; Sharma et al., 2025).

Among these species, H_2O_2 has emerged as a central ROS signal during metal stress because its relatively long half-life and ability to cross biological membranes allow it to convey information between organelles and from the apoplast to the cytosol and nucleus (Foyer & Noctor, 2011; Waszczak et al., 2018). Localized H_2O_2 pulses generated by chloroplasts, mitochondria, peroxisomes or plasma membrane NADPH oxidases trigger oxidation of redox-sensitive cysteine residues in target proteins, leading to reversible changes in enzyme activity, protein–protein interactions and subcellular localization (Domingos et al., 2015; Kolbert et al., 2019). These redox-dependent modifications affect transcription factors, phosphatases and metabolic enzymes, providing a molecular basis for how ROS signatures are translated into adaptive cellular responses (Foyer & Noctor, 2011; Wang et al., 2023). By contrast, excessive production of $O_2\bullet^-$, $\bullet OH$ or $^1O_2$ under severe metal overload overwhelms antioxidant capacity and shifts the balance from signaling, as described in Section 3, toward irreversible oxidative damage and programmed cell death (Gupta et al., 2023; Hasanuzzaman et al., 2020).

The concept of "ROS signatures" describes the specific combinations of ROS species, amplitudes, durations and subcellular localizations that characterize a given stimulus (Mittler, 2017; Waszczak et al., 2018). Heavy metal stress generates ROS signatures distinct from those induced by drought, salinity or pathogen attack, and even among metals, Cd, Pb and Cu elicit different ROS patterns depending on tissue type and genotype (Cuypers et al., 2016; Zhang et al., 2023). These signatures are filtered by redox homeostasis and antioxidant networks (Section 3), which convert ROS fluctuations into biologically meaningful signals. Consequently, moderate and transient ROS elevations promote acclimation, whereas persistent ROS accumulation favors injury, providing a mechanistic explanation for the "double-edged sword" nature of ROS under metal stress (Mittler, 2017; Wang et al., 2025).

4.2. Integration of ROS with Ca^{2+}, MAPK Cascades and Transcriptional Networks

ROS signaling under heavy metal stress is embedded in an interconnected network involving calcium (Ca^{2+}) dynamics, mitogen-activated protein kinase (MAPK) cascades and transcriptional regulation. Early after metal perception, apoplastic and cytosolic ROS accumulation is frequently accompanied by characteristic Ca^{2+} signatures, consisting of transient increases in cytosolic Ca^{2+} that vary in amplitude and duration depending on the metal and tissue involved (Cuypers et al., 2016; Gupta et al., 2023). These Ca^{2+} signals are decoded by calcium-dependent protein kinases and calmodulin-regulated proteins, many of which are themselves redox-sensitive, establishing reciprocal feedback between ROS and Ca^{2+} signaling (Kolbert et al., 2019; Niekerk et al., 2024). The integration of organelle-derived and apoplastic ROS with Ca^{2+} influx, MAPK cascades and transcriptional regulation under heavy metal stress is schematically illustrated in Figure 1.

In parallel, metal-induced ROS activate MAPK cascades, including MPK3 and MPK6, which phosphorylate downstream transcription factors and regulatory proteins that orchestrate stress-responsive gene expression (Cuypers et al., 2016; Kolbert et al., 2019). At the nuclear level, ROS- and MAPK-dependent signals converge on transcription factors from the WRKY, NAC, bZIP, AP2/ERF and related families, regulating genes involved in antioxidant defense, metal transport and sequestration, cell wall remodeling and programmed cell death (Gupta et al., 2023; Sharma et al., 2025). In tolerant genotypes, this ROS–Ca^{2+}–MAPK–TF module promotes rapid activation of detoxification and repair pathways, whereas in sensitive genotypes, dysregulated signaling is often associated with uncontrolled oxidative damage and cell death (Zhang et al., 2023; Sharma et al., 2025).

ROS signaling is further modulated by phytohormone networks, particularly those involving salicylic acid, jasmonic acid, ethylene, abscisic acid and melatonin (Kolbert et al., 2019; Hasanuzzaman et al., 2020). Bidirectional interactions between ROS and phytohormones generate feedback loops that fine-tune signal amplitude and duration, ultimately determining whether plants acclimate to heavy metal stress or succumb to toxicity (Gupta et al., 2023; Zhang et al., 2023; Liu et al., 2024; Wang et al., 2025).

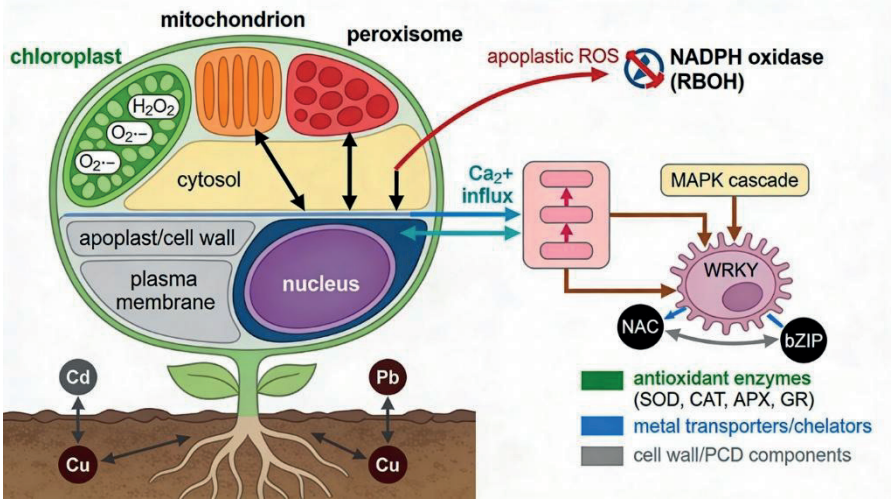

Figure 1. Schematic representation of ROS-based signaling networks in plants exposed to heavy metal stress.

5. ROS–Phytohormone Crosstalk under Heavy Metal Stress

Heavy metal stress induces pronounced reprogramming of plant hormone homeostasis, and many of these alterations are tightly coupled to changes in reactive oxygen species (ROS) production and redox signaling. Rather than acting as independent regulators, ROS and phytohormones form interconnected feedback loops in which ROS influence hormone biosynthesis, transport and signal transduction, while phytohormones reciprocally modulate both ROS-generating and ROS-scavenging systems (Hasanuzzaman et al., 2020; Zhang et al., 2023). This bidirectional crosstalk represents a critical regulatory layer that largely determines whether plants acclimate to metal-contaminated environments or progress toward toxicity-induced injury (Gupta et al., 2023).

A substantial body of evidence indicates that salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are key partners of ROS during heavy metal stress responses. Moderate ROS accumulation often stimulates SA biosynthesis and signaling, which in turn enhances the expression of antioxidant enzymes and defense-related proteins, thereby strengthening detoxification capacity and basal defense mechanisms (Hasanuzzaman et al., 2020). JA and ET are likewise

induced under metal exposure and frequently act in concert with ROS to regulate root growth inhibition, lignification and cell wall remodeling, processes that restrict metal influx and contribute to structural acclimation (Gupta et al., 2023; Sharma et al., 2025). However, excessive or prolonged activation of ROS–SA/JA/ET signaling can shift the response toward growth arrest, senescence and programmed cell death, particularly in metal-sensitive genotypes or under high metal loads (Hasanuzzaman et al., 2020; Zhang et al., 2023), underscoring the quantitative and context-dependent nature of these interactions.

Abscisic acid (ABA) occupies a central position at the interface between ROS signaling and growth regulation under heavy metal stress. Metal-induced ROS bursts frequently promote ABA accumulation and ABA-responsive gene expression, leading to stomatal closure, reduced transpiration and remodeling of root system architecture, collectively limiting metal uptake and long-distance translocation (Gupta et al., 2023; Zhang et al., 2023). In parallel, ABA modulates the expression of antioxidant enzymes and stress-protective proteins, often acting downstream of or in synergy with ROS-activated transcription factors described in Section 4 (Cuypers et al., 2016; Kolbert et al., 2019). In tolerant genotypes, finely tuned ROS–ABA interactions enable plants to balance growth restraint with effective protective adjustments, whereas dysregulated crosstalk in sensitive plants may result in insufficient defense activation or excessive suppression of growth, both of which compromise stress resilience (Sharma et al., 2025; Zhang et al., 2023).

More recently, melatonin and other emerging regulators have been recognized as important components of ROS–phytohormone crosstalk under heavy metal stress. Melatonin exhibits a dual mode of action by directly scavenging ROS and reactive nitrogen species while simultaneously upregulating antioxidant enzymes and metal-chelating systems, thereby reinforcing the redox homeostasis hub described in Section 3 (Kolbert et al., 2019; Zhang et al., 2023). In addition, melatonin interacts with classical hormones such as ABA and JA, modulating their signaling outputs and contributing to improved photosynthetic performance, membrane stability and overall metal tolerance (Hasanuzzaman et al., 2020; Liu et al., 2024). These observations support a model in which melatonin functions not only as an antioxidant but also as a signaling mediator that reshapes the broader ROS–phytohormone network under heavy metal stress.

Overall, ROS–phytohormone crosstalk establishes a multilayered regulatory framework that integrates metal-derived redox cues with developmental and environmental information. Within this framework, ROS signatures generated in specific tissues and subcellular compartments are decoded through hormone-dependent pathways into context-specific outcomes, ranging from transient growth restraint and antioxidant induction to programmed cell death and tissue sacrifice (Cuypers et al., 2016; Mittler, 2017). Elucidating how individual hormones and their combinations interpret ROS signals under different metal regimes will be essential for designing strategies to enhance crop performance and resilience on contaminated soils, for example through targeted manipulation of hormone biosynthesis, signaling components or their redox-sensitive regulators (Gupta et al., 2023; Wang et al., 2025).

6. ROS-Driven Acclimation and Metal Tolerance Strategies

The capacity of plants to withstand heavy metal stress depends not only on restricting metal uptake and detoxifying excess ions, but also on the active deployment of reactive oxygen species (ROS) as signaling molecules that reprogram metabolism, development and defense. As discussed in previous sections, moderate and spatially controlled ROS production can induce antioxidant systems, metal chelation pathways and structural barriers that collectively promote acclimation and enhanced tolerance (Gupta et al., 2023; Liu et al., 2024). In this context, ROS should be viewed not merely as unavoidable by-products of metal toxicity, but as central regulators that integrate metal-derived redox cues into coordinated adaptive responses (Mittler, 2017; Wang et al., 2025).

One major ROS-dependent acclimation mechanism involves the induction and fine-tuning of antioxidant defenses. Under sublethal metal exposure, transient increases in ROS activate redox-sensitive transcription factors and signaling cascades, leading to upregulation of genes encoding superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and key enzymes of the ascorbate–glutathione (AsA–GSH) cycle (Foyer & Noctor, 2011; Ansari et al., 2015). This transcriptional reprogramming is frequently accompanied by elevated levels of low-molecular-weight antioxidants such as ascorbate, glutathione and phenolic compounds, thereby reinforcing the redox homeostasis hub described in Section 3 (Bielen et al., 2013; Gupta et al., 2023). In metal-tolerant genotypes,

these responses are rapid and proportionate, maintaining ROS within a signaling-competent range, whereas in sensitive genotypes delayed or insufficient activation often results in oxidative injury and cell death (Zhang et al., 2023; Sharma et al., 2025).

A second important strategy involves ROS-mediated regulation of metal uptake, transport and compartmentation. ROS signals generated at the root surface and within vascular tissues modulate the expression and activity of transport proteins responsible for metal influx, xylem loading and vacuolar sequestration, including members of the ZIP, NRAMP, HMA and MTP families (Berni et al., 2019; Mansoor et al., 2023). Through their integration with calcium signaling, MAPK cascades and phytohormonal pathways such as abscisic acid and ethylene, ROS influence root architecture, cell wall suberization and lignification, thereby restricting apoplastic metal movement and reshaping root-to-shoot metal distribution (Cuypers et al., 2016; Gupta et al., 2023). At the cellular level, ROS-dependent transcriptional and post-translational control of phytochelatin synthase, metallothioneins and tonoplast-localized transporters promotes chelation and vacuolar sequestration of metals, creating detoxified pools that minimize interference with redox-sensitive metabolic processes (Das & Roychoudhury, 2014; Ali et al., 2025).

ROS signaling further contributes to developmental and morphological adjustments that enhance metal tolerance. Metal-induced ROS modulate root system architecture by affecting lateral root formation and root hair development, often in coordination with auxin and ethylene signaling, thereby optimizing soil exploration while reducing exposure to highly contaminated zones (Kolbert et al., 2019; Gupta et al., 2023). In aerial tissues, interactions between ROS and abscisic acid regulate stomatal behavior, leaf growth and senescence, helping to limit transpiration-driven metal transport and prioritize resource allocation toward survival rather than biomass accumulation (Hasanuzzaman et al., 2020; Zhang et al., 2023). In certain contexts, localized ROS accumulation also promotes the controlled elimination of severely damaged cells, protecting meristems and reproductive organs from extensive metal-induced injury (Liu et al., 2024; Sharma et al., 2025).

At the whole-plant level, variation in ROS handling and ROS-driven signaling underlies the contrasting responses of metal-tolerant and metal-sensitive species, as well as the exceptional performance of hyperaccumulators. Hyperaccumulator plants typically exhibit constitutively high antioxidant capacity, efficient ROS-scavenging systems and robust vacuolar and organellar sequestration mechanisms, allowing them to maintain relatively low oxidative damage despite extremely high internal metal concentrations (Berni et al., 2019; Ali et al., 2025). Comparative analyses indicate that these traits are associated with distinct ROS signatures, redox states and hormone profiles, supporting the view that evolutionary tuning of ROS signaling and redox homeostasis is central to natural metal tolerance (Cuypers et al., 2016; Gupta et al., 2023). Key ROS-related characteristics distinguishing metal-tolerant from metal-sensitive genotypes are summarized in Table 6.1.

Table 6.1. Key ROS-related traits distinguishing metal-tolerant and metal-sensitive plant genotypes

Feature	*Metal-tolerant genotypes*	*Metal-sensitive genotypes*
Basal antioxidant capacity	Generally higher basal activities of SOD, CAT, APX, GR and AsA–GSH cycle enzymes (Berni et al., 2019; Gupta et al., 2023).	Lower basal antioxidant capacity; stronger dependence on late stress-induced activation (Sharma et al., 2025).
Redox buffer pools (AsA, GSH, phenolics)	Larger and more stable pools; GSH/GSSG and AsA/DHA ratios kept within signaling range (Bielen et al., 2013; Ali et al., 2025).	Smaller pools; rapid shift toward oxidized forms and loss of redox control under stress (Bielen et al., 2013; Sharma et al., 2025).
Metal uptake and sequestration	Efficient regulation of ZIP/NRAMP/HMA/MTP transporters; strong vacuolar and organellar sequestration (Berni et al., 2019; Mansoor et al., 2023).	Less controlled metal influx and weaker sequestration, leading to higher cytosolic/organellar free metal (Das & Roychoudhury, 2014; Mansoor et al., 2023).

ROS signatures under metal stress	Moderate, transient ROS increases; compartmentalized signals compatible with acclimation (Cuypers et al., 2016; Liu et al., 2024).	Strong, prolonged ROS bursts; diffuse oxidative stress with extensive macromolecular damage (Gupta et al., 2023; Sharma et al., 2025).
ROS–phytohormone interactions	Better coordinated ROS–ABA/SA/JA/ET/melatonin crosstalk, supporting adaptive growth restraint and defense (Hasanuzzaman et al., 2020; Zhang et al., 2023).	Dysregulated ROS–hormone crosstalk, often leading to excessive growth inhibition or premature cell death (Kolbert et al., 2019; Zhang et al., 2023).
Phenotypic outcome under heavy metals	Higher survival, maintained photosynthetic performance and biomass under given metal dose (Berni et al., 2019; Ali et al., 2025).	Strong growth reduction, chlorosis/necrosis and yield loss at comparatively lower metal levels (Sharma et al., 2025).

Understanding these ROS-driven acclimation strategies provides a robust conceptual framework for improving crop performance on contaminated soils. Targeted manipulation of key components involved in ROS generation, antioxidant networks, metal transport systems and ROS–phytohormone crosstalk offers promising avenues for future breeding and biotechnological approaches aimed at enhancing metal tolerance while minimizing yield penalties, an issue that will be addressed in subsequent sections (Niekerk et al., 2024; Wang et al., 2025).

7. Redox- and ROS-Based Biotechnological Approaches under Heavy Metal Stress

The expanding understanding of redox regulation and ROS signaling under heavy metal stress has opened new opportunities for improving plant performance on contaminated soils through integrated breeding and biotechnological strategies. Rather than indiscriminately suppressing ROS formation, contemporary approaches increasingly focus on optimizing ROS production, scavenging and signaling dynamics, enabling plants to activate efficient acclimation responses while minimizing oxidative damage (Mittler, 2017; Gupta et al., 2023). This conceptual shift underpins current efforts to manipulate antioxidant systems, metal transport processes and ROS–

phytohormone crosstalk using both classical and molecular tools (Liu et al., 2024; Wang et al., 2025).

One widely explored strategy involves enhancing antioxidant capacity and redox buffering. Overexpression of genes encoding key antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), as well as enzymes of the ascorbate–glutathione (AsA–GSH) cycle, has been shown in numerous plant species to alleviate metal-induced oxidative damage and sustain growth under elevated levels of cadmium, lead and other toxic metals (Ansari et al., 2015; Gupta et al., 2023). Likewise, increasing the biosynthetic capacity for glutathione, ascorbate or phenolic antioxidants can stabilize GSH/GSSG and AsA/DHA ratios, thereby reinforcing the redox homeostasis hub described in Section 3 and supporting more robust ROS signaling and detoxification (Foyer & Noctor, 2011; Bielen et al., 2013). However, constitutively elevated antioxidant activity may inadvertently dampen physiologically relevant ROS signals, emphasizing that spatially and temporally regulated expression, rather than blanket upregulation, is essential for maintaining signaling competence (Mittler, 2017; Wang et al., 2025).

A complementary approach targets metal uptake, transport and sequestration pathways that are themselves under redox and ROS control. Manipulation of transporters belonging to the ZIP, NRAMP, HMA, MTP and CAX families can restrict the entry of non-essential metals into sensitive tissues, enhance vacuolar sequestration or redirect metal fluxes toward less vulnerable organs (Berni et al., 2019; Mansoor et al., 2023). For example, increased expression of tonoplast-localized transporters combined with elevated levels of phytochelatins or metallothioneins strengthens vacuolar detoxification and limits metal interference with redox-sensitive processes in chloroplasts and mitochondria (Das & Roychoudhury, 2014; Ali et al., 2025). Integrating such traits with optimized ROS signaling networks holds promise for generating genotypes capable of tolerating higher internal metal loads without incurring severe yield penalties (Cuypers et al., 2016; Gupta et al., 2023).

Emerging technologies further refine these strategies by enabling precise manipulation of redox-regulated targets. High-throughput transcriptomic, proteomic and, in particular, redox-proteomic analyses have identified numerous redox-sensitive proteins involved in signaling, transport and

metabolism that respond dynamically to heavy metal exposure (Noctor et al., 2014; Niekerk et al., 2024). Genome editing technologies offer the possibility to modify such targets with high specificity, for instance by altering redox-sensitive cysteine residues in regulatory proteins, fine-tuning promoter regions to achieve stress-inducible expression, or stacking multiple tolerance-associated alleles within a single genetic background (Liu et al., 2024; Wang et al., 2025). Ultimately, integrating these molecular approaches with conventional breeding and physiological screening for favorable ROS signatures and redox states will be critical for translating fundamental insights into ROS–redox networks into practical solutions for sustainable crop production on metal-contaminated soils (Gupta et al., 2023; Zhang et al., 2023).

8. Conclusion and Future Perspectives

Heavy metal stress presents a multifaceted challenge to plants by simultaneously perturbing cellular redox balance and reshaping reactive oxygen species (ROS) production across multiple compartments. As highlighted throughout this chapter, ROS under these conditions function not only as agents of oxidative damage but also as central signaling molecules that integrate metal-derived cues with antioxidant systems, phytohormone networks and developmental programs (Foyer & Noctor, 2011; Gupta et al., 2023). The outcome of metal exposure—ranging from toxicity and growth inhibition to successful acclimation—ultimately depends on how efficiently plants generate, perceive and decode distinct ROS signatures through redox-sensitive proteins and downstream signaling pathways (Cuypers et al., 2016; Wang et al., 2025).

Future research will need to move beyond the oversimplified assumption that enhanced antioxidant capacity alone confers superior metal tolerance. Instead, emphasis should be placed on elucidating the spatial and temporal dynamics of ROS production and redox states at tissue, cellular and subcellular scales. Recent advances in in vivo redox imaging, genetically encoded biosensors and redox-proteomics are poised to uncover new regulatory nodes, including previously unrecognized redox-sensitive transcription factors, kinases and transporters that coordinate metal uptake, sequestration and detoxification (Noctor et al., 2014; Niekerk et al., 2024). Integrating these approaches with omics-based analyses of natural variation in ROS-related traits will provide a

more nuanced framework for identifying or engineering genotypes with optimized redox homeostasis and ROS signaling under heavy metal stress (Zhang et al., 2023; Liu et al., 2024).

From an applied perspective, embedding redox and ROS concepts into breeding and biotechnological programs offers promising opportunities to enhance crop resilience on contaminated soils while safeguarding yield stability and food safety. Targeted manipulation of antioxidant networks, metal transport systems and ROS–phytohormone crosstalk, guided by systems-level models of redox signaling, may enable the development of cultivars capable of tolerating or even exploiting metal-rich environments, including applications in phytoremediation (Berni et al., 2019; Ali et al., 2025). Ultimately, bridging mechanistic insights with field-oriented strategies will be essential for translating the rapidly expanding knowledge of ROS and redox biology into tangible benefits for sustainable agriculture and environmental management in metal-impacted ecosystems (Gupta et al., 2023; Wang et al., 2025).

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Chapter 10

FATTY ACID COMPOSITION OF TOTAL LIPID, TRIACYLGLYCEROL AND PHOSPHOLIPID FRACTIONS IN GONAD AND LIVER OF FEMALE CARASSIUS AURATUS DURING THE REPRODUCTIVE PERIOD

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1. Introduction

Lipids play a central role in fish physiology, serving not only as major energy reserves but also as essential structural components of cellular membranes and precursors of biologically active compounds. The distribution and composition of lipids in fish tissues are highly dynamic and are influenced by species, habitat, nutritional status, seasonal variation, and reproductive stage. Among these factors, reproduction represents one of the most energetically demanding periods in the life cycle of teleost fishes, during which lipid metabolism is markedly intensified.

Atatürk Dam Lake, located on the Euphrates River, constitutes the largest reservoir in Türkiye and supports a diverse ichthyofauna with substantial fisheries potential. Previous surveys have documented the presence of approximately 28 fish species within this ecosystem (Bozkurt, 1994), highlighting its ecological and economic importance.

Carassius auratus (L., 1758), a member of the family Cyprinidae, is widely distributed across freshwater systems including rivers, lakes, ponds, and stagnant waters. The species exhibits omnivorous feeding behavior, consuming a broad spectrum of food items such as small crustaceans, insect larvae, detritus, and aquatic vegetation (Anon, 2006). Due to its adaptability, *C. auratus* holds significance in capture fisheries, aquaculture, ornamental fish trade, and experimental research, making it a suitable model species for physiological and biochemical studies.

In fish, total lipid content and fatty acid composition vary considerably among tissues and lipid classes. Environmental parameters, particularly water temperature, exert a strong influence on lipid profiles. Decreasing temperatures are generally associated with an increased proportion of unsaturated fatty acids in membrane lipids, a response that contributes to the maintenance of membrane fluidity and cellular function. In this context, phospholipids are primarily involved in structural and regulatory processes, whereas triacylglycerols function predominantly as energy storage molecules.

During the reproductive period, lipid allocation between tissues undergoes pronounced reorganization. Lipids stored in muscle and liver tissues are mobilized and transported to the gonads, where they contribute to

vitellogenesis and oocyte development. The liver plays a pivotal role in this process by acting as both a metabolic processing center and a temporary lipid reservoir. In addition to its metabolic functions, fish liver is recognized as a rich source of nutritionally valuable compounds, including vitamins A and D and long-chain n-3 polyunsaturated fatty acids.

Long-chain polyunsaturated fatty acids (LC-PUFA), particularly docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), and arachidonic acid (AA, 20:4n-6), are essential for normal reproductive function. These fatty acids contribute to membrane architecture, hormone synthesis, and the production of eicosanoids that regulate key reproductive processes. In teleost fishes, phospholipid fractions are typically enriched in LC-PUFA, whereas triacylglycerols mainly reflect dietary inputs and energy storage demands.

Although numerous studies have examined lipid composition in fish muscle, detailed information on the fatty acid profiles of discrete lipid fractions in metabolically active tissues such as liver and gonads remains limited, particularly during the reproductive period. Understanding the tissue- and fraction-specific distribution of fatty acids is essential for elucidating the physiological strategies underlying energy allocation and reproductive success.

Therefore, the present study aimed to characterize the fatty acid composition of total lipid, triacylglycerol, and phospholipid fractions in the gonad and liver tissues of female *C. auratus* during the reproductive season. The findings are expected to contribute to a better understanding of lipid metabolism in cyprinid fishes and provide comparative data for future studies on fish reproductive physiology.

2. Materials and Methods

Female specimens of *C. auratus* were collected from Atatürk Dam Lake (Euphrates River basin, Türkiye) during the reproductive period in March. Fish were captured using commercial fishing nets operated by local fishermen. Immediately after capture, specimens were transported to the laboratory under cold conditions for further processing.

Gonad and liver tissues were carefully excised, and wet weights were recorded prior to analysis. Tissue samples were placed in chloroform–methanol solution (2:1, v/v) and stored at −20 °C until lipid extraction.

Tissue samples were homogenized in chloroform–methanol (2:1, v/v) to extract total lipids following a modified solvent extraction procedure. Non-lipid contaminants, including proteins, carbohydrates, and amino acids, were removed by washing the extracts with 0.88% (w/v) potassium chloride (KCl) solution. The organic phase containing total lipids was recovered and evaporated under nitrogen.

Total lipid extracts were subsequently separated into phospholipid (PL) and triacylglycerol (TAG) fractions using thin-layer chromatography (TLC). The separated lipid bands were visualized, scraped from the plates, and collected for fatty acid analysis.

Fatty acids present in total lipid, PL, and TAG fractions were converted to their corresponding methyl esters (FAMEs) by transmethylation in acidic methanol under reflux conditions. After completion of the reaction, FAMEs were extracted with an organic solvent and concentrated prior to gas chromatographic analysis.

3. Results

The total lipid contents of tissues in female *Cç auratus* are presented in Table 1, while the fatty acid composition of total lipid, TAG, and PL fractions in the gonad and liver tissues is shown in Table 1 and 2.

The total lipid content differed markedly between tissues in female *C. auratus* during the reproductive period. Gonadal tissue exhibited a substantially higher lipid level, reaching 5.13 g per 100 g wet weight, whereas liver tissue contained a considerably lower amount of total lipids (0.55 g per 100 g wet weight) (Figure1).

Analysis of fatty acid composition revealed consistent patterns across total lipid, TAG, and PL fractions in both gonad and liver tissues. Among saturated fatty acids (SFA), palmitic acid (C16:0) was the dominant component in all lipid classes examined. Oleic acid (C18:1n-9) constituted the major monounsaturated fatty acid (MUFA) in both tissues and fractions.

Polyunsaturated fatty acids (PUFA) represented a substantial proportion of the total fatty acid pool. AA (20:4n-6) and EPA (20:5n-3) were consistently detected at relatively high levels in both gonadal and hepatic lipids. In addition, DHA (22:6n-3), a physiologically important n-3 fatty acid, was present in notable amounts across lipid fractions, particularly within phospholipids.

Although the qualitative fatty acid profiles of gonad and liver tissues were broadly similar, quantitative differences were observed between tissues and lipid fractions. Gonadal lipids generally exhibited higher proportions of polyunsaturated fatty acids compared to liver lipids, whereas saturated and monounsaturated fatty acids contributed relatively more to hepatic lipid fractions. Differences were also evident between structural (PL) and storage (TAG) lipids, reflecting tissue-specific distribution patterns during the reproductive period.

Total Lipid Fatty Acid Composition

In the total lipid fraction, ∑SFA accounted for 30.68% in the gonad and 40.37% in the liver. The proportion of ∑SFA was significantly higher in the liver compared to the gonad ($P<0.05$). 16:0 was the dominant SFA in both tissues, with a higher percentage observed in the liver (29.51%) than in the gonad (22.08%). ∑MUFAs were found at 39.44% in the gonad and 32.26% in the liver. 18:1n-9 was the major MUFA in both tissues, representing 30.06% in the gonad and 24.47% in the liver. PUFAs constituted 29.79% of total lipids in the gonad and 27.29% in the liver, with no significant difference between tissues ($P>0.05$). DHA and EPA were prominent n-3 PUFA, particularly in the gonad (13.74% and 4.15%, respectively). The n-3/n-6 ratio was calculated as 3.23 in the gonad and 1.47 in the liver (Figure 2).

Triacylglycerol Fraction

In the TAG fraction, ∑SFA accounted for 35.01% in the gonad and 34.54% in the liver, with palmitic acid (16:0) being the predominant SFA in both tissues. ∑MUFA represented the largest fatty acid group in TAG, comprising 40.42% in the gonad and 40.10% in the liver. 18:1n-9 was the dominant fatty acid in this fraction in both tissues. ∑PUFA levels in TAG were 24.48% in the gonad and 25.22% in the liver. DHA and EPA were present at comparable

levels in both tissues. The n-3/n-6 ratio was 2.98 in the gonad and 2.65 in the liver (Figure 3).

Phospholipid Fraction

In the phospholipid fraction, ∑SFA was higher in the gonad (45.91%) than in the liver (39.50%). 16:0 (32.23%) was the dominant SFA in gonadal phospholipids, whereas 18:0 (16.81%) showed elevated levels in the liver. ∑MUFA accounted for 18.57% in the gonad and 28.23% in the liver. 18:1n-9 was the major MUFA in the phospholipid fraction of both tissues. ∑PUFA levels were notably high in phospholipids, reaching 35.44% in the gonad and 32.22% in the liver. Arachidonic acid (20:4n-6, 13.97%) and DHA (14.88%) were the predominant PUFA in gonadal phospholipids, while EPA (7.11%) and DHA (10.69%) were prominent in liver phospholipids. The n-3/n-6 ratio was calculated as 1.26 in the gonad and 2.19 in the liver (Figure 4).

4. Discussion

The liver represents a central hub of lipid metabolism in teleost fishes, integrating fatty acid uptake, oxidation, modification, and redistribution to peripheral tissues. In particular, it plays a decisive role in supplying long-chain unsaturated and highly unsaturated fatty acids required for growth and reproduction. Previous studies have demonstrated that comparative analysis of lipid profiles in metabolically active tissues such as liver and muscle provides valuable insight into species-specific energy allocation strategies and physiological adaptation (Rincon-Sanchez et al., 1992; Kiessling et al., 2001; Rodriguez et al., 2004; Kaçar and Başhan, 2017).

Lipids constitute a primary energy reservoir supporting somatic growth and reproductive investment. In sexually mature fish, lipid demand increases substantially during the spawning period, reflecting the energetic costs of gonadal maturation and gamete production. Several authors have shown that although the liver functions as a major storage and processing site for lipids destined for reproduction, the immediate energetic requirements of spawning are largely met through mobilization of muscle lipid reserves (Vlaming et al., 1978; Manning & Kime, 1984). A concurrent decline in lipid and fatty acid levels in liver and muscle tissues during the reproductive phase further supports the view that stored lipids are actively recruited to meet

reproductive energy demands (Ackman, 1967; Gill & Weatherley, 1984; Stansby et al., 1990).

While most nutritional studies have traditionally focused on fish muscle due to its dietary relevance, the liver represents a particularly important yet understudied site for long-chain PUFAs. Previous investigations have identified fish liver as a rich source of biologically active lipids associated with visual development, growth regulation, and metabolic health (Njinkoué et al., 2002). In some cultures, fish liver has also been used for medicinal purposes, reflecting its perceived physiological value (Saify et al., 2003). Beyond storage, hepatic lipid fractions—including both structural PL and storage TAGs—exhibit distinct fatty acid signatures that reflect their functional roles within the organism.

Fatty acids play a pivotal role in gonadal development and embryogenesis. During oocyte maturation, fatty acids accumulate within the egg, providing both structural components for developing membranes and metabolic substrates to fuel embryonic growth. Polyunsaturated fatty acids are particularly critical during this process, as they contribute to membrane fluidity and serve as precursors for signaling molecules involved in reproduction (Tocher & Sargent, 1984). Empirical evidence suggests that SFAs, notably 16:0, supply basal metabolic energy during early oocyte formation, whereas monounsaturated fatty acids support later stages of gonadal growth. In contrast, PUFA are preferentially utilized during active reproduction (Huynh, 2007).

During gonadogenesis, fatty acids are mobilized from neutral lipid depots, primarily within adipose tissue, and transported to the liver via circulation. Here, they are incorporated into vitellogenin and subsequently delivered to developing oocytes. Approximately two-thirds of the mobilized fatty acids consist of saturated and monounsaturated species, which are largely catabolized to meet energetic demands. The remaining fraction, enriched in n-3 PUFA, is selectively retained within vitellogenin for incorporation into eggs (Sargent & Henderson, 1995).

Highly unsaturated fatty acids are also essential for maintaining endocrine and reproductive homeostasis. In teleost gonads, arachidonic acid-derived prostaglandins play a regulatory role in steroidogenesis and ovulation

processes (Kellner & Van der Kraak, 1992; Wade & Van der Kraak, 1993). Eicosanoids synthesized from C20 PUFA have been implicated in ovulatory control, hatching success, and early larval development, highlighting their multifunctional importance during reproduction (Mustafa & Srivastava, 1989; Sorbera et al., 1998). Consequently, selective accumulation of C20 PUFA within gonadal tissues appears to reflect adaptive prioritization for reproductive signaling pathways (Jeong et al., 2002).

Sex-specific differences in fatty acid utilization have also been reported. Female fish predominantly rely on saturated fatty acids to meet the energetic requirements of gonadal maturation, whereas males tend to preferentially utilize monounsaturated fatty acids. During vitellogenesis, however, females actively mobilize stored essential fatty acids of both the n-3 and n-6 series, particularly linoleic (18:2n-6) and α-linolenic acid (18:3n-3), underscoring their importance for successful oocyte development (Medford & Mackay, 1978; Cejas et al., 2003).

Overall, the pronounced demand for polyunsaturated fatty acids during gametogenesis highlights their indispensable role in reproductive success. Deficiency of these fatty acids has been associated with impaired gonadal function and reduced fertility, emphasizing their critical physiological significance in teleost fishes (Soivio et al., 1989).

Conclusion

The present study demonstrates that lipid distribution in female *C. auratus* during the reproductive period is strongly tissue- and fraction-dependent. Gonadal tissues contained substantially higher levels of total lipids compared with the liver, reflecting the elevated energetic and structural demands associated with reproduction. Across both tissues, palmitic and oleic acids predominated among saturated and MUFAs, while PUFAs—particularly AA, EPA, and DHA—were prominent components of the lipid pool.

The enrichment of long-chain polyunsaturated fatty acids in phospholipid fractions highlights their importance in membrane structure and reproductive physiology, whereas triacylglycerols primarily reflected energy storage and mobilization. Despite similarities in qualitative fatty acid profiles between gonad and liver tissues, marked quantitative differences indicate selective allocation of fatty acids in response to reproductive requirements.

Overall, these findings provide insight into lipid metabolism and fatty acid utilization during reproduction in cyprinid fishes. The results contribute to a better understanding of tissue-specific lipid dynamics and may serve as a useful reference for comparative studies on fish reproductive biology and nutritional physiology.

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Table 1: Total lipid levels in the gonads and liver of *Carassius auratus.*

Tissue	lipid amount (gr/100gr)
Gonad	5.13±0.33
Liver	0.55±0.01

Table 2- Fatty acids in total lipid, triacylglycerol and phospholipid fractions in the gonads and liver of female *Carassius auratus.*

	Total		Triacylglycerol		Phospholipid	
Fatty acid	Gonad	Liver	Gonad	Liver	Gonad	Liver
14:0	2.58±0.02a	2.33±0.01a	2.82±0.01a	2.84±0.03a	0.97±0.01b	1.00±0.02b
15:0	0.68±0.01a	0.91±0.01b	0.75±0.07a	0.76±0.05a	0.86±0.03b	0.44±0.04c
16:0	22.08±1.21a	29.51±1.36b	25.12±1.02c	24.74±1.67c	32.23±2.20d	20.63±1.05a
17:0	0.18±0.02a	0.75±0.03b	0.66±0.06c	0.65±0.03c	0.81±0.02b	0.62±0.01c
18:0	5.16±0.90a	6.87±0.29a	5.66±0.67a	5.55±0.23a	11.04±0.99b	16.81±0.37c
Σ SFA	**30.68**±2.20a	**40.37**±2.89b	**35.01**±2.12ab	**34.54**±2.65ab	**45.91**±2.88c	**39.50**±1.25b
16:1n-7	7.68±1.05a	5.94±0.99b	6.85±0.45ab	6.79±0.44ab	2.19±0.34c	5.02±0.20b
18:1n-9	30.06±1.21a	24.47±1.47b	31.8±2.09a	31.55±2.06a	15.06±1.05c	21.16±1.24b
20:1n-9	1.70±0.45a	1.85±0.02a	1.77±0.23a	1.76±0.35a	1.32±0.39b	2.05±0.22c
ΣMUFA	**39.44**±1.58a	**32.26**±2.28b	**40.42**±2.34a	**40.10**±1.20a	**18.57**±1.99c	**28.23**±2.05d
18:2n-6	3.22±0.35a	2.29±0.37b	3.04±0.01a	3.26±0.02a	1.19±0.06c	1.66±0.34c
18:3n-3	1.42±0.03a	0.93±0.02b	1.34±0.34a	1.31±0.01a	0.12±0.01c	0.72±0.02b
20:2n-6	0.42±0.02a	0.28±0.03b	0.39±0.01a	0.36±0.03a	0.18±0.01c	0.53±0.01a
20:3n-6	0.35±0.02a	0.28±0.02b	0.33±0.03a	0.34±0.01a	0.35±0.01a	0.46±0.04c
20:4n-6	3.04±0.90a	8.21±0.87b	2.39±0.45c	2.95±0.44a	13.97±0.34d	7.46±0.66b

20:5n-3	4.15±0.02a	3.42±0.01a	3.34±0.08a	3.45±0.90a	3.01±0.99a	7.11±0.35b
22:5n-3	3.45±0.33a	2.01±0.22b	2.86±0.10b	2.78±0.90b	1.74±0.08c	3.59±0.36a
22:6n-3	13.74±1.21a	9.87±1.09b	10.79±1.45b	10.77±1.76b	14.88±1.06a	10.69±1.23b
ΣPUFA	**29.79**±1.66a	**27.29**±1.50a	**24.48**±1.04b	**25.22**±1.28b	**35.44**±2.39c	**32.22**±2.06c
n-3	22.76	16.23	18.33	18.31	19.75	22.11
n-6	7.03	11.06	6.15	6.91	15.69	10.11
n-3/n-6	3.23	1.47	2.98	2.65	1.26	2.19

* Means are the averages of 3 replicates

** Values reported are means ±standard deviation; means followed by different letters in same line are significantly different (p<0.05) by Tukey's test. ***SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

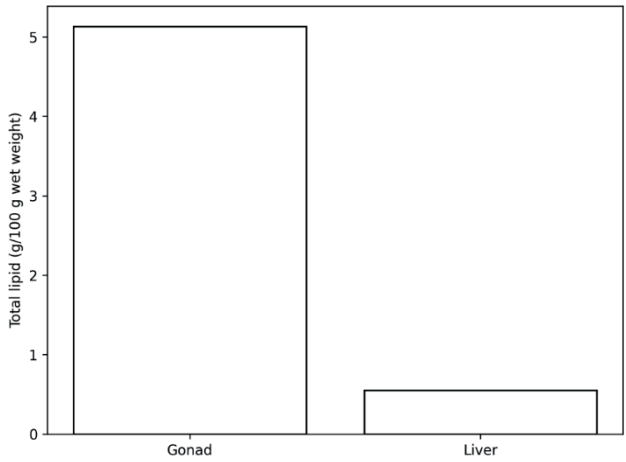

Figure 1

Figure 1. Total lipid content (g/100 g wet weight) in gonad and liver tissues of female Carassius auratus.

Figure 2

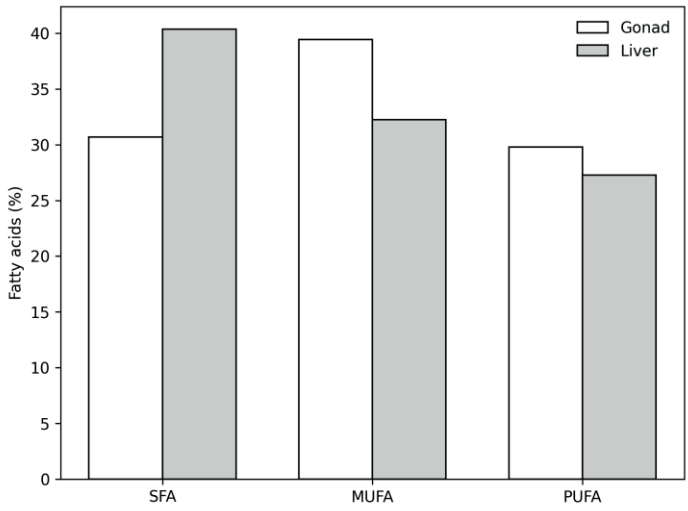

Figure 2. Distribution of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids in the total lipid fraction of gonad and liver tissues of female Carassius auratus.

Figure 3

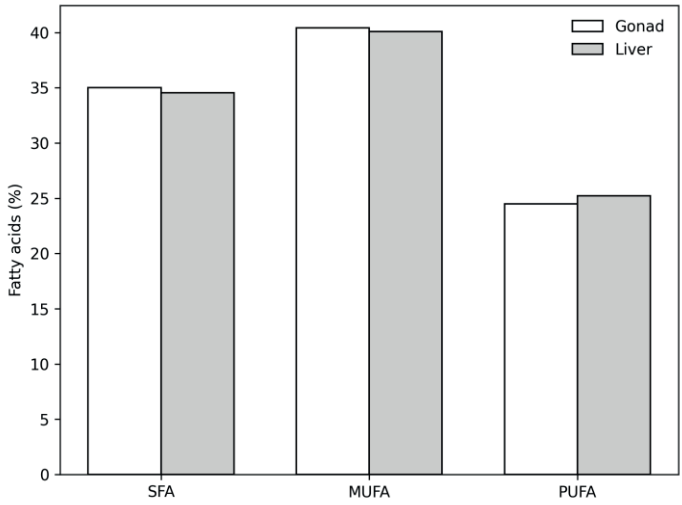

Figure 3. Fatty acid class composition of the triacylglycerol fraction in gonad and liver tissues of female Carassius auratus.

Figure 4

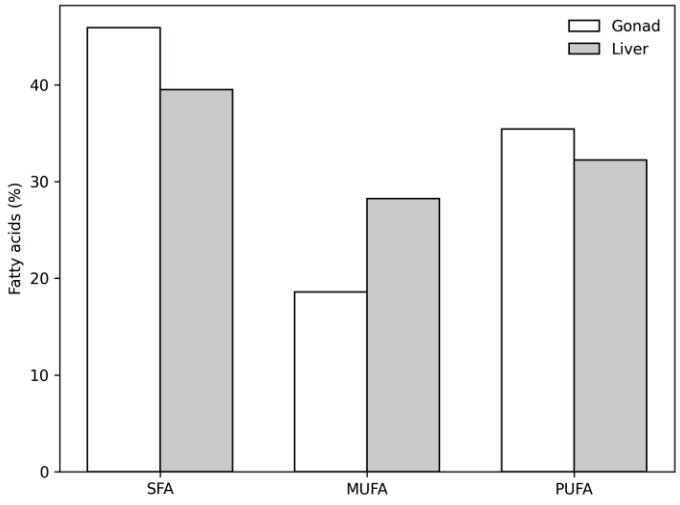

Figure 4. Fatty acid class composition of the phospholipid fraction in gonad and liver tissues of female Carassius auratus.

Chapter 11

AN IN-DEPTH LOOK AT CLIMATE CHANGE THROUGH THE EYES OF SEA TURTLES

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1. INTRODUCTION

Climate change refers to long-term, persistent alterations in the average temperature, precipitation, wind and other atmospheric conditions within the Earth's climate system. These shifts have accelerated in recent decades as a result of natural processes as well as, and particularly due to, human activities. Since the Industrial Revolution, increasing emissions of greenhouse gases such as carbon dioxide, methane and nitrous oxides have disrupted the energy balance of the atmosphere, leading to global warming, which in turn triggers cascading effects across all components of the climate system. Over the past century, the average surface temperature has risen by approximately 1°C, resulting in glacier melt, sea-level rise, droughts and a marked increase in the frequency of extreme weather events. Furthermore, rising temperatures profoundly impact terrestrial and marine ecosystems by altering species' distribution ranges and transforming certain ecosystems in irreversible ways. Climate change represents not only an environmental threat, but also an economic, social and political crisis. Declines in agricultural productivity, disruptions to water resources, loss of biodiversity and increased migration are just some of its multidimensional consequences. Scientists emphasise that mitigating the destructive impacts of climate change requires reducing greenhouse gas emissions, promoting renewable energy sources and developing ecosystem-based adaptation strategies. In this context, combating climate change is not merely an environmental responsibility but a global duty that lies at the heart of sustainable development goals.

Climate change represents a major global risk to the persistence of life on Earth (Sağlam et al., 2008) and has therefore become one of the most prominent issues on the global agenda. It is evident that climate change will generate both direct and indirect effects on numerous species and ecosystems, creating major challenges for the conservation and management of natural resources (Pressey et al., 2007; Newson et al., 2009; Robinson et al., 2009). In this chapter, climate change is examined specifically from the perspective of sea turtles, with a detailed discussion of its impacts on these species.

2. CLIMATE CHANGE

The Earth's climate is undergoing transformation at a pace unmatched in recorded history (Loarie et al., 2009). As a result, species

occupying habitats from the poles to the tropics have begun shifting the timing of seasonal biological events, adjusting their geographical ranges and modifying ecological interactions (Walther et al., 2002; Parmesan & Yohe, 2003). Species respond to climate-related pressures through several mechanisms, which include redistributing their ranges, altering phenotypes via plasticity and undergoing microevolutionary adjustments driven by natural selection (Hulin et al., 2009; Fuentes et al., 2020; Waldvogel et al., 2020).

3. SEA TURTLES

Sea turtles, among the most enigmatic living vertebrates, inhabit all oceans except polar regions and hold significant ecological importance for both marine ecosystem functioning and food webs (Hannan et al., 2007). Male sea turtles remain in the water throughout their lives, while females only emerge onto land for nesting. Their life history is characterised by long-distance migrations between feeding and nesting grounds (Stokes et al., 2015). The evolutionary history of sea turtles dates back roughly 110–120 million years to the Cretaceous Period. Fossil evidence indicates that these animals evolved from terrestrial turtles and adapted to marine environments (Hirayama, 1998). Over time, they developed adaptations such as hydrodynamic shells, flipper-like limbs and salt glands, enabling efficient long-distance oceanic migrations. This group, which diversified throughout the Mesozoic, is among the few reptile lineages to have survived the Cretaceous–Palaeogene mass extinction (Cadena & Parham, 2015). The ancestors of modern sea turtles are believed to have emerged around 65 million years ago, gradually evolving into present-day species. This prolonged evolutionary process accounts for their morphological diversity and global distribution. Consequently, sea turtles are regarded as "living fossils" due to both their ecological roles and their evolutionary history (Spotila, 2004).

Today, sea turtles are classified into two families: Cheloniidae and Dermochelyidae, represented by seven species—one belonging to Dermochelyidae and six to Cheloniidae (Pritchard, 1996). These species are *Dermochelys coriacea, Eretmochelys imbricata, Chelonia mydas, Lepidochelys olivacea, Lepidochelys kempii, Caretta caretta* and *Natator depressus* (Figure 1).

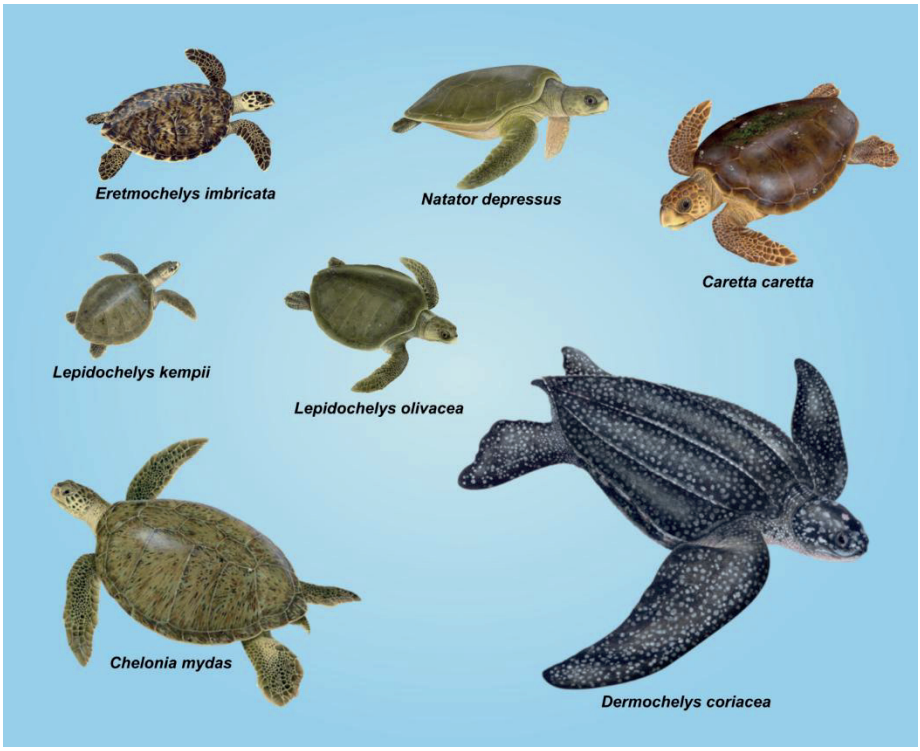

Figure 1. *The seven extant sea turtle species. Of these species, Dermochelys coriacea belongs to the family Dermochelyidae, whereas the remaining six species belong to the family Cheloniidae*

These species face a suite of natural and anthropogenic pressures. Natural threats include disease and predation (Hawkes et al., 2009; Heithaus, 2013), while human-driven impacts encompass bycatch (Putman et al., 2020; de la Hoz Schilling et al., 2023), poaching (Joseph et al., 2019), pollution from plastics (Wilcox et al., 2018; Solomando et al., 2022) and light pollution on nesting beaches (Truscott et al., 2017). Climate change, however, stands out as a dominant and pervasive threat due to its effects on all life stages and both terrestrial and marine habitats (Fuentes et al., 2011; Laloë et al., 2017; Turkozan et al., 2021).

4. CLIMATE CHANGE FROM THE PERSPECTIVE OF SEA TURTLES

Climate change is among the most significant environmental threats affecting sea turtles' life cycles, reproductive success and population stability. Figure 2 summarises major effects of climate change on sea turtles. Foremost

among these is temperature-dependent sex determination. Even slight increases in sand temperature can shift hatchling sex ratios dramatically towards females, threatening the long-term viability of populations by disrupting reproductive balance (Sarı & Kaska, 2015; Şirin & Başkale, 2024). Additionally, rising sea temperatures and sea-level rise further intensify beach erosion, increase nest loss and elevate embryo mortality (Patrício et al., 2019). Climate change also influences sea turtles' foraging ecology and migration dynamics. Shifts in sea temperatures alter migration routes and the distribution of essential food resources such as seagrass beds (Hawkes et al., 2009). Thus, climate change represents a multidimensional stressor that threatens not only nesting beaches but also the species' global ecological balance. Accordingly, climate-based conservation strategies—such as nest shading, controlled incubation and habitat management—are becoming increasingly important.

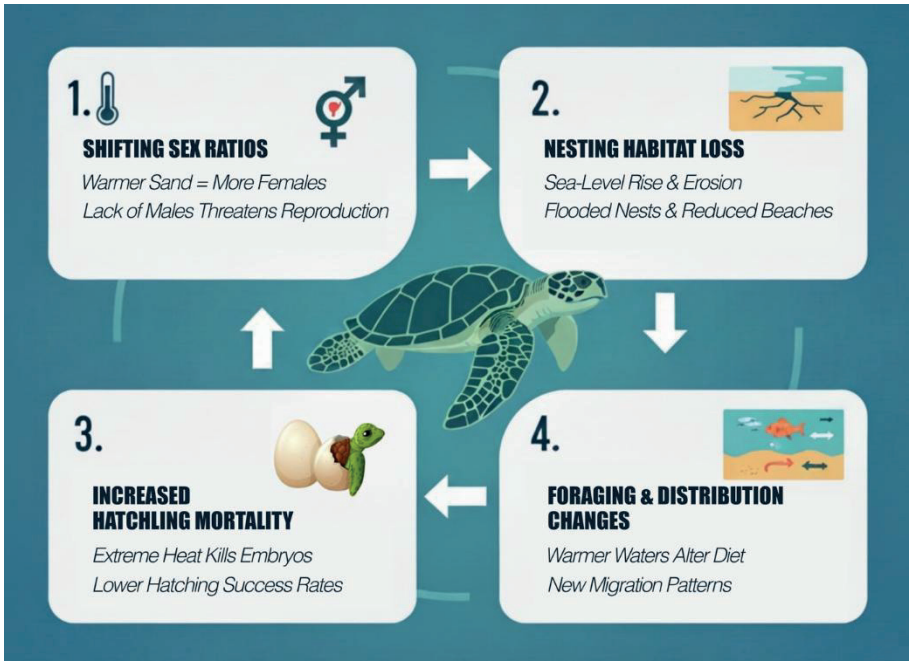

Figure 2. *Summary of the effects of climate change on sea turtles*

4.1. Effects of Climate Change on Beach Habitats

Eggs and hatchlings constitute the most sensitive life-history stages for sea turtles. Although they already face natural threats from predators and

disease, climate change introduces additional pressures that threaten their developmental success and long-term viability.

Incubation temperature plays a decisive role in determining hatchling sex. The pivotal temperature at which an equal sex ratio is produced is approximately 29°C (Mrosovsky et al., 2002; Wibbels, 2003). However, even slight deviations from this value can heavily bias sex ratios. A minor temperature rise typically produces female-skewed clutches, whereas a slight cooling favours male hatchling (Wibbels, 2003). The potential for global warming to skew hatchling sex ratios towards females raises serious concerns for the future of sea turtles. Indeed, research conducted over the past fifty years demonstrates that the effect of climate change on sex ratios is among the greatest risks to the sustainability of these species. Decades of research indicate that many nesting beaches already produce predominantly female hatchlings, and male outputs are projected to decline even further.

Temperature also affects embryonic viability. An increase of 1°C may reduce hatch success by around 25% (Howard et al., 2014). Once temperatures exceed ~35°C, embryo mortality becomes widespread (Howard et al., 2014; Cavallo et al., 2015; Laloë et al., 2017). The mechanisms behind thermal-induced mortality may be associated with reduced oxygen availability or developmental stress (Ackerman, 1980; Packard et al., 1977).

Humidity and precipitation influence nest conditions as well. Moderate rainfall can improve nest success in arid environments (Montero et al., 2019), but storm-related downpours often result in nest flooding and widespread embryonic death (del Monte-Luna et al., 2023). Increasing storm frequency and rainfall intensity are therefore predicted to create additional challenges for many populations (Seidel et al., 2008).

Thermal conditions during incubation influence hatchling performance. Crawling speed—critical for avoiding predators—declines when temperatures fall outside the 28–32°C optimal range (Booth, 2017; Liles et al., 2019). Similarly, elevated incubation temperatures negatively affect swimming performance (Drake & Spotila, 2002), with evidence linking developmental temperatures to physiological traits that influence oceanic survival (Cavallo et al., 2015).

Sea-level rise, exacerbated by human coastal development, is shrinking nesting habitats, flooding nests and increasing nest loss (Poloczanska et al., 2009). Some beaches cannot shift inland due to topographic barriers, further constraining available nesting space. Several studies predict that nesting habitat could decline by up to 85% for some species by the end of the century (Patrício et al., 2019; Fuentes et al., 2020). While some high-energy beaches may create new nesting areas for species such as *D. coriacea*, these gains are expected to be limited.

4.2. Effects of Climate Change on Marine Habitats

In marine environments, climate change affects sea turtles primarily through alterations in foraging ecology and migration patterns. Rising water temperatures and changes in ocean circulation can shift migration timing, redirect migratory pathways and modify the locations of productive foraging grounds (Poloczanska et al., 2009; Patrício et al., 2021).

Sea turtles rely on long-distance migrations—some exceeding 10,000 km—to access suitable foraging and nesting sites (Godley et al., 2008). They are believed to possess sophisticated navigational abilities that enable them to relocate these areas consistently (Bentivegna et al., 2007; Georges et al., 2007). However, shifts in prey distribution and warming seas have been linked to changes in migratory routes (Sherrill-Mix et al., 2008).

Adult turtles may experience increased stranding risk due to altered ocean temperatures (Winton et al., 2013). Although global warming implies overall warming trends, certain regions may paradoxically experience localised cooling, resulting in more frequent cold-stunning events (Griffin et al., 2019). In the Mediterranean, migration routes may remain relatively stable, but thermal changes are expected to reduce food availability for some species (Petsas et al., 2023).

Hatchlings depend heavily on ocean currents for dispersal; altered current systems can prevent them from reaching food-rich developmental habitats (Wildermann et al., 2017; DuBois et al., 2020). Sea-surface warming is predicted to shift suitable foraging areas poleward for species such as *C. caretta* and *D. coriacea* (Chaloupka et al., 2008; Sherrill-Mix et al., 2008). Increasing temperatures are driving *L. kempii* into higher latitudes, where sudden cold events pose significant threats (Griffin et al., 2019).

Climate change may reduce productivity in some foraging regions while increasing it in others, potentially expanding habitat suitability for certain populations such as *D. coriacea* (Witt et al., 2007). However, warming waters in the Mediterranean may reduce the ability of *C. mydas* to utilise dormancy-like strategies during food-limited periods (Hochscheid et al., 2007). Coastal foraging areas remain particularly vulnerable to storms and cyclones, which can induce mass strandings.

Acidifying oceans further drive changes in the distribution and abundance of key marine species, consequently affecting sea turtle foraging opportunities. Satellite telemetry and ecological modelling reveal that climate-driven environmental shifts may force *C. caretta* northwards in both the Atlantic and Mediterranean, while restricting *D. coriacea* foraging ranges in the Pacific (Patrício et al., 2021).

4.3. Effects of Climate Change on Diseases

Infectious diseases, already intensified by environmental stress, are becoming more prevalent under changing climatic conditions (Fey et al., 2015). Rising sea temperatures, altered storm patterns and ocean acidification all influence pathogen dynamics, placing sea turtles at increased risk (Tracy et al., 2019).

One of the best-known diseases, fibropapillomatosis, causes tumour-like growths that can impede movement and may result in mortality (Jones et al., 2016). Warmer waters are expected to enhance tumour proliferation (Foley et al., 2005). Fungal infections also represent a growing threat; *Fusarium solani*, in particular, is widespread in nests and can significantly reduce embryo survival (Sarmiento-Ramírez et al., 2014). While fungal presence does not always lead to mortality, susceptibility increases when embryonic immune function is compromised (Gleason et al., 2020). Sea-level rise can force turtles to nest at higher densities, potentially accelerating pathogen spread. Ultimately, climate change reduces immune competence, increases pathogen ranges and exposes turtles to novel diseases across terrestrial and marine habitats.

5. CONCLUSION

Climate change represents a complex and multidimensional pressure that influences every stage of the sea turtle life cycle, from the early embryonic period to adulthood. Elevated sand and seawater temperatures, shifts in oceanographic conditions, habitat degradation and the increasing frequency of extreme weather events collectively reshape key biological processes such as sex determination, embryonic survival, migratory behaviour and foraging strategies. These disruptions alter population structures, reduce reproductive output and compromise the long-term resilience of many sea turtle populations across the globe.

Nesting beaches—already under intense pressure from coastal development, tourism and artificial lighting—are becoming increasingly unstable. Rising temperatures skew sex ratios towards females, while sea-level rise and intensified storm activity reduce the availability and quality of nesting habitat. In many regions, the combined effect of erosion, inundation and habitat fragmentation may result in the loss of suitable nesting areas within decades, creating demographic bottlenecks that could take centuries to recover from.

Marine habitats are undergoing similarly profound changes. Altered current systems, ocean warming and shifts in food-web dynamics are driving modifications in migration timing and influencing the distribution of critical foraging grounds. While some species may temporarily benefit from increased productivity in particular regions, most are predicted to encounter shrinking or relocating habitats, forcing them to travel further or adapt to rapidly changing environmental conditions. These shifts not only heighten energetic costs but may also increase exposure to fisheries, marine debris and vessel strikes.

The disease dimension of climate change adds yet another layer of complexity. Warmer waters and changing precipitation patterns can accelerate the spread of pathogens such as *Fusarium* spp. and exacerbate diseases like fibropapillomatosis, particularly in individuals with compromised immune systems. The cumulative impact of weakened immunity, increased pathogen prevalence and higher nesting density—caused

by reduced beach area—poses an escalating threat to both hatchlings and adult turtles.

Given the magnitude and interconnectivity of these threats, conservation strategies must be adaptive, long-term and grounded in climate projections. Protecting and restoring nesting habitats, implementing interventions such as nest shading and controlled incubation, safeguarding foraging grounds and reducing additional anthropogenic pressures are essential components of an integrated management plan. Equally important is the development of region-specific monitoring programmes that can track how populations respond to changing conditions and inform targeted conservation actions.

Ultimately, the survival of sea turtles in a rapidly changing climate depends on the global community's ability to reduce greenhouse gas emissions, curb habitat destruction and invest in nature-based, climate-resilient conservation approaches. Without coordinated international action, the ecological and evolutionary legacy of these ancient marine reptiles may face irreversible decline.

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Chapter 12

THE EFFECTS OF FUNGI ON ELEMENT ACCUMULATION AND TROPHIC NETWORKS

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Element Cycles and the Position of Fungi in Ecosystems

In natural ecosystems, the circulation of elements occurs as a result of the complex and multifaceted interaction of biotic and abiotic processes. Essential elements such as carbon, nitrogen, phosphorus, sulfur, and metals are constantly exchanged between soil, water, atmosphere, and living organisms, ensuring the continuity of ecosystem functioning. In these cycles, fungi, particularly due to their direct contact with soil and organic matter, occupy a strategic position in the fixation, transformation, and redistribution of elements. The ability of their large surface area mycelial networks to reach different microhabitats makes fungi key biological actors in regulating element Dynamics (Treseder ve Lennon, 2015; Mayer et al., 2021). Regional studies conducted in Türkiye demonstrate that fungal community composition, particularly macrofungi and myxomycetes, shows high habitat specificity and functional diversity, which directly influences decomposition capacity and element cycling dynamics at the ecosystem scale (Ergul et al., 2005a; Ergul et al., 2005b Ergül and Akgül, 2011; Akata et al., 2018). Fungi have the capacity to accumulate both essential nutrients and potentially toxic elements in their biomass by taking them up from the environment. This feature transforms fungi from passive element acceptors into active regulators that directly influence the bioavailability and mobility of elements within the ecosystem. Through element accumulation, fungi have a decisive impact on nutrient balance, primary productivity, and ecosystem resilience to environmental stresses (Treseder and Lennon, 2015). Filamentous fungi decompose complex organic compounds such as lignin and cellulose, releasing carbon, nitrogen, and phosphorus, a process central to the soil organic matter cycle. While decomposer fungi are characterized by rapid carbon mineralization and CO_2 release, some fungal groups are identified as "stress tolerants," producing structures such as resinous compounds, β-glucans, and trehalose, which decompose more slowly and support long-term carbon accumulation. These differences in community composition directly affect the size and persistence of soil carbon stocks (Treseder and Lennon, 2015; Mayer et al., 2021). Global studies have revealed a strong correlation between fungal biomass and persistent soil carbon bound to reactive minerals. Hyphae–mineral interactions accelerate the decomposition

of organic matter while simultaneously enabling fungal necromass to bind to mineral surfaces, contributing to the persistence of carbon in the soil for thousands of years. This dual effect demonstrates that fungi play simultaneous roles in both carbon release and carbon sequestration processes (Wang et al., 2025). There are significant differences in element accumulation among fungal guilds. Soil saprotrophic fungi support fast-cycle systems with relatively high nitrogen and phosphorus content in their stem tissues, while wood-rotting fungi are associated with slower decomposition and carbon accumulation due to high C:N ratios and low nitrogen content. Ectomycorrhizal fungi, on the other hand, support long-term cycles by directing carbon to the root zone with their relatively low nitrogen and phosphorus content (Liu et al., 2020; Mayer et al., 2021; Pánek et al., 2024).

When evaluated in terms of the bioavailability of nutrients, ectomycorrhizal fungi can release nitrogen and phosphorus from organic matter through enzymatic systems, Fenton chemistry, and priming effects. Phosphorus solubility is increased by the secretion of organic acids and phosphatases, while water and nutrient uptake by plants is effectively supported through mycelial networks (Sağlıker and Darıcı, 2007; Nehls and Plassard, 2018; Liu et al., 2020). Arbuscular mycorrhizal fungi, even under conditions of global warming and nitrogen accumulation, can regulate plant carbon-nitrogen-phosphorus stoichiometry, simultaneously increasing both nutrient turnover rate and nutrient stability (Mei et al., 2024; Ma et al., 2025). In aquatic ecosystems, decomposer fungi regulate nutrient flow by intensely immobilizing nitrogen and phosphorus on leaf litter. In these systems, while the C:N ratio of fungal biomass generally remains constant, the C:P and N:P ratios show flexibility depending on the ambient phosphorus level; excess phosphorus is stored through lux uptake, creating a buffering effect in nutrient cycles (Yaşar et al., 2009; Gulis et al., 2017). In arid and nutrient-poor microhabitats, fungal networks transport water, carbon, and nitrogen, ensuring the maintenance of bacterial activity and indirectly supporting microbial processes and element cycling (Worrich et al., 2017). Fungi also significantly influence the behavior of metals and phosphates within the ecosystem. By exposing rocks and minerals to bioaerobic conditions, fungi facilitate the dissolution of metals and phosphates, and can stabilize these elements through binding on the cell surface, intracellular deposition, or biomineralization processes. Biomineralization mechanisms, such as the formation of calcium oxalate

and metal phosphate, affect both plant nutrition and the immobilization of pollutants by altering the mobility and toxicity of metals (Gadd, 2017; Gadd, 2021). Biomineralization processes occurring in the root zone can contribute to the retention of toxic metals in the rhizosphere and the re-vegetation of contaminated areas. This demonstrates that fungi play functional roles not only in nutrient cycling but also in ecosystem restoration and the management of contaminated areas (Gadd, 2017; Gadd, 2021). From a nitrogen fixation perspective, fungal networks can immobilize nitrogen in biomass, limiting the release of mineral nitrogen despite microfauna grazing. This feature indicates that nitrogen fixation, rather than nitrogen mineralization, is dominant in fungal-dominant systems and constitutes an important mechanism in the long-term regulation of ecosystem productivity (Ghaderi et al., 2025). As soil fertility increases, the rise in saprotrophic fungi and oxidizing enzyme activity leads to increased tree growth and nutrient cycling rates, along with a decrease in carbon and nitrogen stores in the organic layer (Mayer et al., 2021). Conversely, excessive nitrogen accumulation can shift fungal communities towards more stress-tolerant species, leading to weakened decomposition capacity and altered carbon sequestration dynamics. However, fungal species tolerant to extreme conditions such as high temperature, salinity, acidity, and pollution can maintain decomposition and nutrient cycling even under these stresses, contributing to the long-term resilience of forest ecosystems (Sağlıker and Darıcı, 2005; Treseder and Lennon, 2015; Gadd, 2017; Moore et al., 2021; Talal et al., 2025).

Mechanisms of Element Uptake and Accumulation in Fungal Cells

Element accumulation in fungi occurs through the simultaneous and coordinated operation of numerous cellular and molecular mechanisms. These mechanisms are generally classified into two main groups: passive and active processes, based on metabolic energy requirements. Passive processes involve the binding of elements to cell wall components without energy expenditure, while active processes involve the controlled uptake and distribution of elements into the cell via specific carrier proteins, ion channels, and intracellular compartmentalization. The combined operation of these two processes enables fungi to survive

even in heavy metal-rich or contaminated environments (Robinson et al., 2021; Gajewska et al., 2022; Shi et al., 2024; Zbieralski et al., 2024). Field investigations on wild mushrooms reveal that heavy metal accumulation is frequently associated with elevated oxidative stress markers, indicating a tight physiological link between elemental uptake and redox regulation in fungal cells (Akgül et al., 2016; Sevindik et al., 2017a).The fungal cell wall is one of the essential structures forming the first line of defense in elemental accumulation. Thanks to the carboxyl, phosphate, hydroxyl, and amine groups found in chitin, glucans, melanin, proteins, and polysaccharides, the cell wall acquires a strong anionic surface property and can adsorb metal ions without requiring metabolic energy. This passive biosorption mechanism has been reported to be effective for numerous metals such as copper, zinc, cadmium, lead, and mercury; FTIR and SEM-EDX analyses have shown that metals interact with amino, carbonyl, hydroxyl, and phosphoryl groups on the cell wall (Chen et al., 2019; Gajewska et al., 2022; Šebesta et al., 2022; Kurniawan et al., 2023; Gori et al., 2025). Among cell wall components, melanin holds a privileged position in terms of its metal-binding capacity. Melanin, which is abundant in black mushrooms, acts as a strong binder for highly toxic metals such as Cr(VI), forming an effective external barrier that limits the diffusion of metal ions into the cell. Similarly, it has been shown that exocellular polysaccharides produced by mushrooms can bind more than 60–80% of zinc, cadmium, and lead in a short time and rapidly reduce the bioavailability of metals in the environment (Medina-Armijo et al., 2024; Jaroszuk-Ściseł et al., 2025). In addition to metals held at the cell surface by passive mechanisms, fungi can control elemental balance at a more subtle level through active uptake and intracellular regulation processes. Metal ions are taken up into the cell via specific carrier proteins and channels; these carriers include high and low affinity systems for iron, ZIP-like carriers for zinc, and various divalent cation carriers. These active uptake processes are tightly regulated at the gene level depending on environmental metal abundance and cellular requirements (Kosman, 2003; Shi et al., 2019; Robinson et al., 2021). Instead of being left free in the cytoplasm, metals taken up into the cell are rapidly complexed with binding molecules. Metallothioneins, glutathione, and other low molecular weight chelators convert metal ions into less toxic forms, thus limiting cellular damage. These complexation mechanisms contribute to the preservation of metabolic integrity of fungal cells by creating a

rapid and flexible response system to metal stress (Robinson et al., 2021; Gajewska et al., 2022). Long-term control of excess metal accumulation is mostly achieved through vacuolar sequestration. Vacuolar transporters such as Zrc1/Cot1 for zinc, Ccc1/VIT1 family proteins for iron, and divalent cation transporters for metals such as cadmium and lead play a critical role in this process. The proton gradient created via V-ATPase facilitates the transport of metals into the vacuole, reducing cytoplasmic toxicity. TEM-EDX-based studies support this multi-compartment storage strategy by confirming that copper, lead, zinc, and cadmium accumulate in both the cytoplasm and vacuoles (Sorribes-Dauden et al., 2020; Robinson et al., 2021; Shi et al., 2024). When these cellular processes are considered together, it is seen that element accumulation in fungi is organized as a multi-layered defense network. The basic components of this network include immobilization via the cell wall, melanin, and exocellular polysaccharides on the outside; complexation with chelator proteins and sequestration in vacuoles on the inside; and, when necessary, removal of metal ions from the cell via efflux pumps. This holistic organization makes fungi extremely resilient organisms both in naturally metal-rich niches and in contaminated soils and waters (Robinson et al., 2021; Gajewska et al., 2022; Shi et al., 2024; Zbieralski et al., 2024). Consequently, element accumulation achieved by fungi through a combination of passive and active mechanisms enables not only cellular survival but also the control of environmental metal mobility and toxicity. These characteristics make fungi powerful bioremediation agents in the biological containment of heavy metal pollution and environmental remediation strategies; they offer a strategic biological infrastructure for the sustainable management of element cycles (Kurniawan et al., 2023; Tamjidi et al., 2023; Jamir et al., 2024; Gori et al., 2025).

Accumulation of Essential and Toxic Elements by Fungi

Elements accumulated by fungi are generally classified into two main groups based on their functional properties: essential and toxic. Essential elements such as potassium, calcium, magnesium, iron, zinc, and copper are crucial for maintaining fungal metabolism and play fundamental roles in enzyme catalysis, energy production, osmotic balance, and the maintenance of cell wall and membrane integrity. Fungi can efficiently absorb these elements, even when present in low concentrations in the environment, thanks to their high-affinity uptake systems,

and store them in their biomass (Gerwien et al., 2018; Alselami and Drummond, 2023). Experimental cultivation studies have shown that substrate composition significantly affects mineral accumulation and oxidative balance in edible mushrooms, highlighting the importance of environmental conditions in determining elemental profiles (Sevindik et al., 2016; Gürgen et al., 2020).Essential metals function as necessary cofactors for metabolic processes at low concentrations, but can exhibit toxic effects in cases of excessive accumulation. Therefore, fungi have developed homeostatic mechanisms that tightly control metal uptake and intracellular distribution. Studies in various fungal species reveal that calcium, potassium, magnesium, iron, and zinc become significantly enriched in mycelium and sporocarps depending on environmental conditions, and that this accumulation is regulated by species-specific metabolic strategies (Gerwien et al., 2018; Ye et al., 2022; Krzesłowska et al., 2025; Mleczek et al., 2025; Wikandari et al., 2025). Experimental studies conducted under controlled conditions have shown that mushroom biomass can be enriched with essential elements. In filamentous mushrooms grown for food purposes, it has been reported that the addition of iron, zinc, and calcium to the culture medium can increase the levels of these minerals in mushroom biomass by 5 to 13 times, and that growth performance is not negatively affected when applied at appropriate doses. These findings indicate that mushrooms have significant potential in terms of nutrient enrichment and functional food production (Wikandari et al., 2025). In contrast, toxic elements such as cadmium, lead, mercury, arsenic, and chromium pose a potential stressor for fungi. However, many fungal species are able to retain these elements in their cell wall components, vacuoles, or biomass by forming complexes with metal-binding proteins and organic acids, thus limiting their toxic effects. This detoxification capacity enables fungi to survive in heavy metal-rich natural niches or contaminated environments (Domka et al., 2019; Han et al., 2019; Dhalaria et al., 2020; Khalid et al., 2021; Priyadarshini et al., 2021; You et al., 2021; Ahammed et al., 2023; Zhao et al., 2024; Tariq and Farhat, 2025). Mycorrhizal and endophytic fungi play a particularly important role in regulating the mobility of toxic elements within the ecosystem. Arbuscular mycorrhizal fungi can restrict the mobility and entry of metals such as cadmium and lead into root tissues by forming complexes with these elements through dense hyphal networks they create around the roots and the glomalin they secrete. This immobilization mechanism

constitutes an effective first line of defense for plants exposed to metal stress (Dhalaria et al., 2020; Zhao et al., 2024). At the intracellular level, mycorrhizal fungal vesicles and other fungal vacuoles provide high-capacity storage areas for heavy metals, preventing the transport of toxic elements into plant tissues. Findings based on meta-analyses show that arbuscular mycorrhizal fungi reduce the concentration of potentially toxic elements in plant shoots in contaminated soils by an average of 24%, while slightly increasing metal accumulation in the roots. Depending on the species and metal concentration, it has been reported that the transport of elements such as zinc and cadmium from the root to the shoot can be suppressed or controlled (You et al., 2021; Han et al., 2021; Chen et al., 2023; Ma et al., 2026;). The plant protective effects of mycorrhizal fungi are not limited solely to physical immobilization. These fungi contribute to limiting metal-induced oxidative stress by stimulating antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase, as well as glutathione and phytochelatin systems. Similarly, endophytic fungi enhance plant metal tolerance by binding metals, retaining them in cell walls and organelles, and promoting plant growth; in this respect, they function as a "biological shield" similar to mycorrhizal fungi (Domka et al., 2019; Dhalaria et al., 2020; Khalid et al., 2021; You et al., 2021; Zhao et al., 2024; Ma et al., 2026). When evaluated at the ecosystem scale, the ability of wood-decaying and soil fungi to accumulate both essential and toxic metals at high levels leads to the formation of characteristic "element profiles" specific to species and decay type. These profiles determine the speed and direction of metal cycles, exerting powerful influences on the nutrient balance and environmental security of ecosystems. Thanks to processes such as sorption, bioaccumulation, biotransformation, and precipitation of heavy metals, fungi stand out as powerful biological agents for mycoremediation applications (Priyadarshini et al., 2021; Chaurasia et al., 2023; Gori et al., 2025; Krzesłowska et al., 2025; Mleczek et al., 2025).

The Effect of Element Accumulation on Ecosystem Functioning and Trophic Networks

Fungal element accumulation profoundly affects ecosystem functioning not only through micro-scale cellular processes but also through trophic networks. Elements accumulated in fungal biomass can be transported to higher trophic levels by invertebrates, insects, and microfauna that feed

on the fungi; this process supports the balanced distribution of essential elements within the ecosystem, while also carrying the potential risk of biomagnetism in the case of toxic elements. In this respect, fungi shape ecosystem dynamics as "bridge organisms" that both store elements and transport them along biotic networks (Crowther et al., 2012; Hauer et al., 2025; Junggebauer et al., 2025). Beyond nutrient transfer, fungal biomass represents a source of bioactive compounds with antioxidant, antimicrobial, and neuroprotective properties, which may indirectly influence trophic interactions and organismal fitness across food webs (Mohammed et al., 2019; Sevindik et al., 2021; Sevindik et al., 2017b). Fungal-derived element accumulation in soil ecosystems has indirect but powerful effects on plant productivity and species composition by determining the speed and direction of nutrient cycles. Ectomycorrhizal and arbuscular mycorrhizal fungi can intensively store nitrogen and phosphorus in their mycelial networks; arbuscular mycorrhizal fungal mycelium, in particular, has been shown to constitute a significant nutrient reservoir underground with a phosphorus content more than 20 times higher than that of plant tissues. This mycelial deposition leads to the redistribution of C–N–P stocks on a fine spatial scale, indirectly altering soil heterogeneity, and consequently plant species composition and productivity (Waring et al., 2016; Sağlıker et al., 2025; Xu et al., 2025; Zhang et al., 2025). Saprotrophic fungi decompose complex organic substances such as lignin and cellulose, mineralizing carbon and nitrogen and making these elements accessible again to plants and other microorganisms. This process is considered one of the fundamental mechanisms controlling the balance between soil carbon storage and solubility. Fungal-induced changes in decomposition rates determine both the efficiency of the nutrient cycle and the long-term dynamics of soil organic matter stocks (Treseder and Lennon, 2015; Wang et al., 2025; Xu et al., 2025). The transfer of elements accumulated in fungal biomass along trophic networks occurs primarily through soil fauna. Soil invertebrates such as columbali, mites, worms, and diplopods meet a large portion of their energy and nutrient requirements from fungal-derived carbon and nitrogen; in many soil systems, fungal channels stand out as the primary energy pathway. These organisms accelerate the redistribution of nutrients both horizontally and vertically by transporting the elements they take up with fungal hyphae to higher trophic levels via their biomass and excrement (Crowther et al., 2012; Junggebauer et al., 2025; Hauer et al., 2025). In aquatic ecosystems, element accumulation

and transfer by fungi occur through unique trophic pathways defined as "mycoloops". It has been shown that parasitic fungi (e.g., chytrids) infecting phytoplankton transfer nitrogen obtained from host diatoms to their spores, and the consumption of these spores by zooplankton results in approximately 14% daily nitrogen transfer. This process, which reduces the carbon/nitrogen ratio across trophic levels, contributes to the creation of a higher-quality food source for zooplankton (Sánchez Barranco et al., 2020). The Mycoloop mechanism enables the return of elements, normally locked in large and inedible phytoplankton cells, to zooplankton and higher trophic levels. In this way, fungi play a central role in the functioning of aquatic food webs by regulating the distribution of elements and energy flow between the water column and sediment (Sánchez Barranco et al., 2020). When evaluated in terms of toxic elements, fungi are known to be able to concentrate various elements in their tissues, including trace metals (such as Cu, Ni, Cd, Zn). However, it has been reported that these metals do not exhibit a generalized and obligatory biomagnetism pattern in multitrophic terrestrial ecosystems; rather, transfers are species- and element-specific. This indicates that fungal-derived element accumulation has a more complex and context-dependent dynamic within the ecosystem context (Tibbett et al., 2021; Uygun et al., 2025). However, under certain conditions, particularly in contaminated areas or when pathogenic fungi are transported by vectors such as microplastics, the transfer of toxic elements and other harmful components to higher trophic levels can increase the risk of secondary toxicity and disease at the local level. Therefore, fungal element accumulation and trophic transfer are processes that need to be carefully considered in terms of ecosystem health and environmental risk assessments (Gkoutselis et al., 2021; Tibbett et al., 2021).

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Chapter 13

THE ROLE OF FUNGI IN NATURAL SYSTEMS

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Mushrooms as Silent Controllers of Matter and Energy Cycles

The sustainability of ecosystems depends on the balanced and uninterrupted circulation of matter and energy between biotic and abiotic components. Fungi, which are central to these cycles, play a critical role, particularly in the transformation and re-bioavailability of organic matter. Through their heterotrophic feeding strategies, fungi decompose dead plant and animal tissues, converting complex biopolymers into simpler compounds, thereby releasing nutrients and facilitating the transfer of energy to different trophic levels within the ecosystem. In this respect, fungi are considered among the fundamental biological regulators that determine the speed and direction of ecosystem metabolism (Wilhelm et al., 2019; Meng et al., 2022; Li and Dong, 2025; Vieira et al., 2025). The ability of fungi to break down resistant plant compounds such as lignin, cellulose, and hemicellulose makes them indispensable actors in the global carbon cycle. In particular, while lignin, due to its chemical structure, can only be degraded to a limited extent by most bacteria, white rot fungi can effectively break down this polymer through powerful oxidative enzyme systems. Enzymes such as lignin peroxidase, manganese peroxidase, and laccase, produced by species like Phanerochaete chrysosporium, directly contribute to the reintroduction of carbon stored in plant residues back into the biosphere cycle by facilitating the mineralization of lignin-derived aromatic compounds (Miyauchi et al., 2017; Wilhelm et al., 2019; Kato et al., 2024; Vieira et al., 2025). The degradation of cellulose and hemicellulose is also largely carried out by ascomycete and basidiomycete fungi, which play a key role in the microbial degradation of lignocellulosic structures using a broad repertoire of CAZyme such as hydrolases and oxidoreductases. Studies in terrestrial forest soils and compost systems reveal that the rate of lignocellulose degradation is strongly dependent not only on fungal abundance but also on the structure of fungal-bacterial interaction networks and the presence of low-abundance but functionally critical "keystone" taxa (Miyauchi et al., 2017; Wilhelm et al., 2019; Meng et al., 2022; Datta, 2024; Deep et al., 2025; Vieira et al., 2025). This indicates that degradation processes occur through a multi-actor and network-based dynamic. The role of fungi in the carbon cycle is not limited to terrestrial systems but also shows significant effects in marine ecosystems. It has been reported that macroalgal endophytic fungi contribute to the breakdown of cellulose and, to some extent, lignin under both oxic and anoxic conditions, thereby directly affecting the marine carbon cycle (Perkins et al., 2021; Lankiewicz et al., 2023; Lankiewicz et al., 2025). These findings indicate that the functions of fungi in carbon mineralization and organic matter transformation should be considered on an inter-ecosystem scale.

In addition to the carbon cycle, fungi also play crucial roles in the nitrogen and phosphorus cycles. Ectomycorrhizal and arbuscular mycorrhizal fungi support both individual plant performance and primary productivity

at the ecosystem scale by increasing nitrogen and phosphorus uptake by plants. The main mechanisms of this contribution include the enzymatic uptake of organic nitrogen and the increased solubility of phosphorus through the secretion of organic acids and phosphatases (Zak et al., 2019; Liu et al., 2020; Mei et al., 2024; Shukla et al., 2025). In parallel, these fungal groups also indirectly regulate the storage and stability of soil organic carbon (Zak et al., 2019; Ma et al., 2025; Shukla et al., 2025). The average C:N:P ratio of fungal biomass being approximately 250:16:1 indicates that fungi carry a strong stoichiometric signal on soil nutrient limitation and decomposition rates. This unique stoichiometric structure plays a critical role in determining which nutrient element will be limiting in fungal decomposition processes (Gulis et al., 2017; Zhang and Elser, 2017; Pánek et al., 2024; Uygun et al., 2025). Similarly, it has been reported that fungal communities developing on leaf litter in aquatic ecosystems immobilize and store phosphorus in particular thanks to their relatively stable C:N ratios, thereby shaping nutrient flow and the structure of food webs (Gulis et al., 2017; Deep et al., 2025; Li and Dong, 2025). When ecosystem functioning is evaluated in the context of climate change, mycorrhizal fungi are identified as "hidden decomposers" that control soil organic matter cycling, CO_2 emissions, plant productivity, and the carbon sink capacity of forest ecosystems. Nitrogen accumulation and climatic variables alter the structure, abundance, and enzyme production profiles of AM and EcM communities, reorganizing decomposition rates and carbon storage dynamics (Zak et al., 2019; Han et al., 2020; Mei et al., 2024; Ma et al., 2025). Therefore, a decrease in fungal diversity or the loss of specific functional guilds can lead to slower lignocellulose decomposition, accumulation of organic matter, and serious bottlenecks in nutrient cycling (Wilhelm et al., 2019; Meng et al., 2022; Li and Dong, 2025; Vieira et al., 2025).

The Structural Effect of Fungi in the Construction of Earth Architecture

Soil is not merely a passive medium formed by the aggregation of mineral particles; it is a dynamic and living system constantly reshaped by biological processes. Although the role of fungi in the formation of the physical and chemical properties of this system has long been considered secondary, mycelial networks stand out as one of the fundamental structures ensuring the functional integrity of soil. These networks, composed of long, thin, and branched hyphae, bind soil particles together, promoting aggregate formation and directly influencing the spatial organization of soil structure (Tisdall, 1994; Lehmann et al., 2020; Wei et al., 2024; de Goede et al., 2025). Specifically, arbuscular mycorrhizal (AM) fungi significantly increase the formation and binding energy of water-resistant macroaggregates through their hyphal networks and the glomalin-like proteins they secrete. Studies have shown that this effect persists even under drought conditions, and that hyphal length

and glomalin content are among the critical variables explaining aggregate stability (Yang et al., 2017; Ji et al., 2019; Cheng et al., 2021; Ji et al., 2024). These findings reveal that fungal-derived biomolecules play a central role in the stability of soil structure. The contribution of filamentous fungi to soil architecture occurs not only through biochemical but also through physical mechanisms. The physical enveloping and binding of soil particles by hyphae, and the secretion of external polymers such as polysaccharides, glycoproteins, and hydrophobics, make the fungi essentially producers of "biological cement." Experimental studies conducted with numerous isolates have revealed that increases in hyphae biomass and density are positively correlated with aggregate formation and stability (Lehmann et al., 2020; Angulo et al., 2024; Wei et al., 2024; de Goede et al., 2025). These aggregate structures formed by fungi not only increase the mechanical strength of the soil but also significantly affect its water-holding capacity and pore structure. Aggregates formed with the contribution of glomaline and hydrophobic components regulate the soil's water potential, allowing moisture to be retained for longer periods, and simultaneously improve gas exchange and hydraulic conductivity through the reorganization of pore connections. These processes directly contribute to reducing the risk of erosion and increasing soil functionality (Yang et al., 2017; Ji et al., 2019; Cheng et al., 2021; Ji et al., 2024; Liu et al., 2024; Yu et al., 2024; Wei et al., 2024; de Goede et al., 2025). When evaluated at the soil-plant interface, the role of mycorrhizal fungi becomes even more prominent. AM fungi, thanks to their extra-root hyphal networks, increase the uptake of water, especially phosphorus, nitrogen, and potassium, by plants; increases of up to approximately 50% in plant biomass and root volume have been reported under drought conditions. In parallel, root architecture and hormone balance change in mycorrhizal plants, and drought tolerance is strengthened by the expansion of root surface area (Yaşar et al., 2009; Cheng et al., 2021; Chandrasekaran, 2022; Wang et al., 2023; Liu et al., 2024). The effects of fungal activity on soil architecture also have significant implications for microbial ecology. Mycelial networks and fungal aggregates create microhabitats for bacteria and other microorganisms, allowing for increased soil microbial diversity. It has been reported that bacterial-fungal interaction networks are more complex and dense, particularly in large and stable aggregates, and that these structures simultaneously support multiple nutrient cycles. This clearly demonstrates that fungi play a central regulatory role in maintaining both the structural and functional integrity of soil (Wagg et al., 2019; Lehmann et al., 2020; Liao et al., 2020; de Goede et al., 2025).

The Regulatory Power of Fungi in Interactive Networks of Organisms

Ecosystems are built upon multi-layered and dynamic networks of interactions between different groups of living organisms. Fungi, at the center of these networks, determine the direction and strength of biotic relationships

through both direct and indirect mechanisms. Their symbiotic relationships with plants provide mutual benefit in terms of nutrient sharing and tolerance to environmental stresses, while pathogenic interactions constitute an effective control mechanism in limiting population sizes and regulating species composition. The simultaneous existence of these opposing forms of interaction makes fungi key actors in shaping ecosystem balance. Mycorrhizal fungi increase the uptake of essential nutrients, primarily phosphorus and nitrogen, from plant roots in exchange for carbon, while also improving water supply and stress tolerance. This reciprocal interaction directly affects not only individual plant performance but also the structure of plant communities and the symbiotic strategies of species. The transmission of nutrient and chemical signals between plants through mycorrhizal networks facilitates seedling establishment and contributes to balancing interspecies competition by supporting niche differentiation in the soil. In contrast, plant pathogenic fungi can lead to significant structural changes in ecosystems. Suppression or elimination of key plant species through infection can result in a decrease in plant diversity and chain reactions of disruptions in trophic networks. This demonstrates that fungi reshape the composition of plant communities through their pathogenic roles and indirectly affect ecosystem stability. Therefore, the balance between mutualistic and pathogenic interactions of fungi is considered a fundamental element in determining the long-term stability of ecosystems (Tedersoo et al., 2020; Mishra et al., 2024).

The regulatory effects of fungi are not limited to plant communities but also extend to fundamental ecosystem processes such as nutrient cycling, carbon sequestration, and water balance. Changes in competition and decomposition rates between different mycorrhizal types can increase or decrease the amount of carbon stored in the soil. Simultaneously, fungal-induced interactions contribute to the reorganization of community structures by altering the ecological niches and competitive strengths of plant species (Sağlıker and Darıcı 2007; Tedersoo et al., 2020; Bahram and Netherway, 2022; Mishra et al., 2024; Wang and Kuzyakov, 2024). In this respect, fungi stand out as functional regulators influencing ecosystem processes at multiple scales. Fungal-bacterial interactions also constitute a fundamental component of ecosystem functioning. These interactions occur in almost all habitats across a wide spectrum ranging from mutualism to intense competition, affecting numerous processes from biochemical cycles and soil fertility to plant and animal health and disease suppression. Particularly in soil and rhizosphere environments, bacteria gain an advantage in simpler carbon sources, while fungi excel in the decomposition of complex organic matter; this competition determines decomposition rates and the direction of nutrient cycling (Deveau et al., 2018; Wagg et al., 2019; Zhou et al., 2022; Wang and Kuzyakov, 2024). The chemical dimension of these interactions is constituted by the secondary

metabolites produced by both groups. Compounds such as antibiotics, toxins, and signaling molecules reshape the structure of microbial communities by regulating competition and communication between microorganisms. These metabolites produced by fungi lead to the suppression or promotion of specific bacterial groups, enabling the enrichment of bacterial taxa that support plant growth or suppress pathogens. Thus, fungal-bacterial interactions play a central role in the continuity of ecosystem functions and the maintenance of biotic balance (Netzker et al., 2015; Wagg et al., 2019; Alam et al., 2021; Bahram and Netherway, 2022; Elhamouly et al., 2022; Lapiere and Richard, 2022; Berrios et al., 2024; Wang and Kuzyakov, 2024).

Fungus-Based Mechanisms in Ecosystem Resilience and Stress Adaptation

Natural ecosystems function under the simultaneous pressure of multiple stressors such as climate change, environmental pollution, and habitat loss. The resilience of ecosystems to these stresses depends not only on the preservation of species numbers but also on the maintenance of functional diversity and resilience. In this context, fungi are among the essential biotic components that enhance the adaptation capacity of ecosystems to environmental changes; they contribute to the overall resilience of the system both through direct physiological mechanisms and through their interactions with other groups of organisms (Cheng et al., 2021; Liu et al., 2022; Madouh and Quoreshi, 2023; Sağlıker et al., 2025). Many fungal species possess the ability to develop exceptional tolerance to heavy metals, pesticides, and various toxic compounds. This tolerance is based on multiple cellular mechanisms such as the binding of metal ions in the cell wall, biosorption and bioaccumulation processes, reduction or transformation of metal ions, vacuole or cell wall sequestration, and active outpumping. These physiological strategies not only enable fungi to survive under stressful conditions but also allow them to assume a protective function at the ecosystem level by reducing the bioavailability of toxic compounds in the environment (Priyadarshini et al., 2021; Robinson et al., 2021). In particular, some endophytic fungal species can significantly increase the production of metallothioneins, glutathione, melanin, and antioxidant enzymes under heavy metal stress, converting metals into less toxic organic forms. These biochemical responses both prevent damage to fungal tissues and reduce the impact of metal stress on host plants. These characteristics reveal that fungi are of strategic importance to ecosystems not only in terms of stress tolerance but also in terms of their bioremediation potential (Priyadarshini et al., 2021; Robinson et al., 2021; You et al., 2025).

The contributions of fungi to ecosystem resilience are not limited to heavy metal tolerance but are also clearly evident under abiotic stresses

such as drought and salinity. Under drought conditions, the development of extraradical hyphal networks, the regulation of aquaporins, and the accumulation of osmoprotectants, thereby increasing water uptake and water use efficiency, are among the main mechanisms by which fungi support plant production. Similarly, under salinity stress, the regulation of the potassium/sodium ratio and the retention of toxic ions in the root zone demonstrate the plant physiology-balancing effect of fungi (Sağlıker and Darıcı, 2005; Bahadur et al., 2019; Begum et al., 2019; Diagne et al., 2020; Cheng et al., 2021; Sharma et al., 2021; Liu et al., 2023). Plant-fungal symbioses, particularly through arbuscular mycorrhizal fungi (AMFs), play a central role in the development of resilience at the ecosystem scale. AMFs establish symbiotic relationships with a large proportion of terrestrial plants, increasing the uptake of phosphorus, nitrogen, water, and micronutrients; thereby improving plant growth and physiological performance under both normal and stressful environmental conditions. Strengthening antioxidant defense systems, osmotic regulation, maintaining phytohormone balance, and regulating gene expression are fundamental processes in the face of drought, salinity, temperature extremes, and heavy metal stress (Bahadur et al., 2019; Begum et al., 2019; Diagne et al., 2020; Cheng et al., 2021; Sharma et al., 2021; Wahab et al., 2023; Nie et al., 2024; Radi et al., 2025). When evaluated at the ecosystem scale, systems with high species diversity of arbuscular mycorrhizal fungi and other root endophytes are found to be more resilient to environmental stresses and have a stronger post-stress recovery capacity. Maintaining plant productivity during drought periods and rapid recovery after drought clearly demonstrates the ecosystem-stability-enhancing effect of fungal diversity. Global analyses show that the richness of saprophytic and mycorrhizal fungi, in particular, strongly supports the temporal stability and drought resistance of plant production (Cheng et al., 2021; Liu et al., 2022; Madouh and Quoreshi, 2023). Similar trends are observed in aquatic ecosystems. In freshwater systems, a decrease in fungal species richness weakens the rate of organic matter decomposition and biomass accumulation, while high fungal diversity enables these processes to continue even under environmental stress conditions. These findings reveal that fungi not only adapt to current environmental conditions but also play a decisive role in preparing ecosystems for future uncertainties (Graca et al., 2024).

From Natural Ecosystems to a Sustainable Future: The Ecological Services of Mushrooms

The roles of fungi in ecosystems are not limited to natural biochemical processes but also encompass numerous ecological services vital to human societies. These services include the decomposition of organic waste, the biological remediation of polluted soil and water environments, and the

maintenance of nutrient cycles. Saprotrophic fungi, in particular, are almost the only group of organisms capable of breaking down lignocellulosic structures, converting dead plant and animal tissues into minerals and CO_2, thus forming the basis of soil fertility and ecosystem productivity (Heilmann-Clausen et al., 2015; Treseder and Lennon, 2015; Zanne et al., 2020). In soil ecosystems, fungi play key roles in nitrogen, phosphorus, and carbon cycles, contributing to the maintenance of soil health and the sustainment of climate regulatory services. Strengthening soil aggregation, promoting organic matter accumulation, and long-term carbon sequestration are direct outcomes of fungal activity. These processes not only improve soil structure but also regulate greenhouse gas emissions and buffer ecosystems against climate change (Treseder and Lennon, 2015; Větrovský et al., 2020; Adnan et al., 2022; Akter et al., 2025). Mycorrhizal fungal-plant symbioses constitute one of the most common and effective examples of ecosystem services provided by fungi. Currently, more than 250,000 plant species are known to have symbiotic relationships with mycorrhizal fungi; these associations significantly improve growth, stress tolerance, and survival success by increasing water and nutrient uptake by plants. It has been shown that these symbioses maintain plant productivity and strengthen sustainability in agricultural and forestry systems under environmental stress conditions such as drought and salinity (Powell and Rillig, 2018; Adnan et al., 2022; Martin and van Der Heijden, 2024). The ecosystem services of fungi are not limited to terrestrial systems; they also play significant and indispensable roles in freshwater ecosystems. Aquatic fungi maintain nutrient cycles through leaf decay and organic matter decomposition, transferring energy flow to higher trophic levels and supporting the functioning of aquatic food webs. These processes also enhance the natural "self-cleaning capacity" of water, contributing to the preservation of water quality and the continuity of clean water ecosystem services (Seena et al., 2023; Graca et al., 2024). The ecosystem services offered by fungi are becoming increasingly important for human-centered bioeconomic applications. Fungus-based technologies are becoming widespread in areas such as food production, drug development, biofuel and biomaterial (e.g. myco-leather and biocomposites) production. In addition, the strong enzymatic and metabolic capacity of fungi offers high potential for the bioremediation of contaminated soil and water environments and is becoming a fundamental component of nature-based solution approaches (Zanne et al., 2020; Seena et al., 2023; Akter et al., 2025; Case et al., 2025). The relationship between fungal diversity and ecosystem functions stands out as a decisive factor for the long-term stability of ecosystems. In systems with high richness in decomposer and mycorrhizal fungi, it has been shown that multiple ecosystem functions are simultaneously strengthened and plant biomass increases. This reveals that fungal diversity provides a fundamental biological infrastructure supporting ecosystem multifunctionality (Treseder and Lennon, 2015; Powell and Rillig, 2018; Liu et al., 2022; Runnel et al., 2025).

Furthermore, fungal diversity plays a critical role in maintaining ecosystem resilience to climate extremes. Specifically, ecosystems with high decomposer fungal richness have been reported to exhibit more stable plant productivity and faster ecosystem recovery during periods of drought. These findings demonstrate that fungi are key organisms supporting ecosystem stability under climate change conditions (Liu et al., 2022; Akter et al., 2025). Conversely, disruptions and biodiversity losses in fungal communities pose a serious threat to essential ecosystem services such as food security, soil health, and climate regulation. Declining fungal biodiversity can lead to slowed decomposition processes, disruptions in nutrient cycling, and increased vulnerability of ecosystems to environmental stressors (Heilmann-Clausen et al., 2015; Frac et al., 2018; Fang et al., 2023; Akter et al., 2025). Therefore, fungi should be considered not only as "complementary" elements of ecosystems, but also as habitat providers, indicators of ecosystem processes, and strategic tools for nature-based solutions. The systematic integration of fungi into conservation biology and sustainable environmental management approaches emerges as a critical requirement for the preservation of ecosystem functioning (Heilmann-Clausen et al., 2015; Fang et al., 2023).

Global databases and functional feature-based approaches developed in recent years make it possible to correlate fungal diversity with environmental responses and ecosystem services. Such holistic research frameworks contribute to a deeper understanding of fungal ecology and enable the effective use of fungal-based biological infrastructure for a sustainable future (Prieto et al., 2019; Větrovský et al., 2020; Zanne et al., 2020; Bahram and Netherway, 2022).

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