University of Zagreb

Faculty of Science
Department of Biology

Iva Jurčević Šangut

IN VITRO BIOLOGICAL ACTIVITY AND TISSUE-SPECIFIC AND SEASONAL PROFILING OF BIFLAVONOIDS IN GINKGO (*Ginkgo biloba* L.)

DOCTORAL DISSERTATION

Zagreb, 2026

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Supervisor: Asst. Prof. Dunja Šamec, PhD

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Sveučilište u Zagrebu

Prirodoslovno-matematički fakultet
Biološki odsjek

Iva Jurčević Šangut

BIOLOŠKA AKTIVNOST *IN VITRO* I TKIVNO SPECIFIČNO TE SEZONSKO PROFILIRANJE BIFLAVONOIDA U GINKU (*Ginkgo biloba* L.)

DOKTORSKA DISERTACIJA

Mentorica: doc. dr. sc. Dunja Šamec

Zagreb, 2026.

This doctoral dissertation was made at the University North, Department of Food Technology in Koprivnica, under the supervision of Assistant Professor Dunja Šamec, PhD, as part of the Doctoral Programme in Biology at the University of Zagreb, Faculty of Science, Department of Biology, within the project funded by the Croatian Science Foundation - “The Role of Biflavonoids in Plants: *Ginkgo biloba* L. as a Model System” (project number UIP-2019-04-1018).

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Prvo, veliko hvala mentorici, doc. dr. sc. Dunji Šamec na prilici da budem sudionicom ovog velikog, dugog i šarolikog istraživačkog puta, koji je zasigurno objema ostavio značajan trag, kako na osobnom tako i profesionalnom planu. Bilo je zaista obogaćujuće biti dijelom ove životne etape obilježene ginkom.

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S ljubavlju,

Iva

SUPERVISOR INFORMATION

Assistant Professor Dunja Šamec, PhD

Dunja Šamec was born on October 15, 1984, in Varaždin, where she completed the First Gymnasium in 2003. The same year, she enrolled at the Faculty of Food Technology and Biotechnology at the University of Zagreb. She pursued a double major and, in 2008, graduated with two master's degrees, in Food Engineering and Nutrition. During her studies, she received the Rector's award, the Dean's award, and a scholarship from the Biotechnical Foundation. Since 2009, she has been employed at the Division of Molecular Biology at Ruđer Bošković Institute in Zagreb, where she obtained her PhD in the field of biology in 2013 within the Doctoral Programme in Molecular Biosciences. She spent a total of three years in research training at international institutions in the USA, Germany, and the Czech Republic, specializing in plant metabolomics and plant molecular biology. From 2014 to 2016, she conducted her postdoctoral training at Washington State University in Pullman, WA, USA, within the framework of the MSCA Newfelpro Fellowship, focusing on the metabolomics of plants and plant-derived foods. Since 2017, she has held a permanent position as a Research Associate at the Ruđer Bošković Institute in the field of biology, where she has led UFK projects and participated in several Croatian Science Foundation, national, and EU projects. Since February 2021, she has been employed as an Assistant Professor at the Department of Food Technology at University North, where she teaches several courses and is currently serving as the head of the Department of Food Technology. She is the project leader of a Croatian Science Foundation project, an institutional project funded through the NPOO program, an ERASMUS+ KA220 project, and also serves as the lead at her institution for a project funded under the Competitiveness and Cohesion programme. She has published 75 papers in international scientific journals, most of which are ranked in Q1, with the majority as first or corresponding author. According to Google Scholar, her publications have been cited 5421 times, with an *h*-index of 33. She is a regular reviewer for numerous international journals. Her achievements have been recognized with the Croatian State Science Award, the Influential Croatian Women Awards, and multiple Director's Awards from the Ruđer Bošković Institute. She is also active in science popularization and, in the last two years, has been listed among the world's top 2% most cited scientists in the field of food science, plant biology and agronomy according to the Stanford University ranking.

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University North, Department of Food Technology, Trg dr. Žarka Dolinara 1, Koprivnica

Abstract

Dimeric forms of flavonoids occur in certain plant species, although their roles in plants remain understudied. Ginkgo (*Ginkgo biloba* L.) is one of the plant species that accumulate 3′,8″-biflavones. The objectives of this doctoral thesis were to evaluate the *in vitro* biological activity of 3′,8″-biflavones in comparison with their monomeric subunits, to optimize their extraction, separation, and quantification procedures, to determine their content and distribution in different ginkgo tissues, and to monitor their dynamic changes throughout the entire growing season. For this purpose, an HPLC-DAD method was developed, a detailed analysis of the tissue distribution of biflavonoids was performed, and their seasonal dynamics were systematically described for the first time. The results showed that dimerization does not lead to a universal enhancement of biological activity, but rather selectively modulates specific biological effects. The biflavonoids exhibited pronounced, yet species- and concentration-dependent antifungal activity, as well as significant inhibitory potential toward acetylcholinesterase and enzymes involved in glycemic regulation, whereas their direct antioxidant activity was limited. It was also established that biflavonoids accumulate more intensively toward the end of the growing season, making yellow autumn leaves a phytochemically valuable plant material.

Keywords: HPLC-DAD method, biflavonoids, ginkgo, amentoflavone, bilobetin, ginkgetin, isoginkgetin, sciadopitysin

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Doktorska disertacija

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Sažetak

Dimerni oblici flavonoida pojavljuju se u određenim biljnim vrstama, iako njihova uloga u biljkama još uvijek nije dovoljno istražena. Ginkgo (*Gingko biloba* L.) je jedna od biljnih vrsta koja akumulira 3′,8″-biflavone. Ciljevi ovog doktorskog rada bili su: procijeniti *in vitro* biološku aktivnost 3′,8″-biflavona u usporedbi s njihovim monomernim podjedinicama, optimizirati postupke njihove ekstrakcije, razdvajanja i kvantifikacije, odrediti njihov sadržaj i distribuciju u različitim tkivima ginka te pratiti dinamičke promjene tijekom cijelog vegetacijskog razdoblja. U tu svrhu razvijena je HPLC-DAD metoda, provedena je detaljna analiza tkivne raspodjele biflavonoida te je po prvi put sustavno opisana njihova sezonska dinamika. Rezultati su pokazali da dimerizacija ne uzrokuje univerzalno pojačanje biološke aktivnosti, već selektivno modulira pojedine biološke učinke. Biflavonoidi su iskazali izraženu, ali vrsno i koncentracijski ovisnu antifungalnu aktivnost te značajan inhibitorni potencijal prema acetilkolinesterazi i enzimima uključenima u regulaciju glikemije, dok je njihova izravna antioksidativna aktivnost bila ograničena. Utvrđeno je i da se biflavonoidi intenzivnije akumuliraju pri kraju vegetacije, zbog čega žuti jesenski listovi predstavljaju fitokemijski vrijedan biljni materijal.

Ključne riječi: HPLC-DAD metoda, biflavonoidi, ginkgo, amentoflavon, bilobetin, ginkgetin, isoginkgetin, sciadopitysin

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1. Introduction

1.1. Flavonoids - chemical basis, structural diversity, and biological role in plants

Flavonoids represent the largest and most extensively investigated group of specialized plant metabolites within the broader class of polyphenols. To date, more than 9000 individual compounds have been identified, which are further classified into subgroups according to specific modifications of the basic structure (Zhuang et al., 2023). Flavonoids possess a basic C6–C3–C6 skeleton composed of two aromatic rings (rings A and B) connected by a three-carbon bridge, forming an oxygenated heterocyclic ring (ring C). This 15-carbon flavone skeleton serves as the primary structural framework from which all flavonoid subclasses arise.

Structural diversity among flavonoids originates from variations in the oxidation state of the C ring, the mode of attachment of the B ring to the C ring, and the nature and position of substituents on the A and B rings (Dwivedi et al., 2017). Together, these structural features and post-biosynthetic modifications underpin the extraordinary chemical diversity of flavonoids. Based on the characteristic oxidation pattern and heterocyclic C-ring configuration, flavonoids are conventionally classified into six major subclasses: flavones, flavonols, flavanones, flavanols (catechins), anthocyanidins, and isoflavones (Figure 1). The aglycone represents the fundamental flavonoid scaffold, which can undergo a range of common and largely universal structural modifications, including hydroxylation, *O*-methylation, glycosylation (most frequently with glucose, rhamnose, or rutinose), sulfation, and glucouronidation, all of which strongly influence solubility, stability, and biological activity (Tang et al., 2025; Ku et al., 2020; Šamec et al., 2022, 2021). In addition, less common but structurally significant modifications such as prenylation and dimerization give rise to more complex flavonoid derivatives, including biflavonoids (Zou et al., 2025; Šamec et al., 2022; Wen et al., 2022).

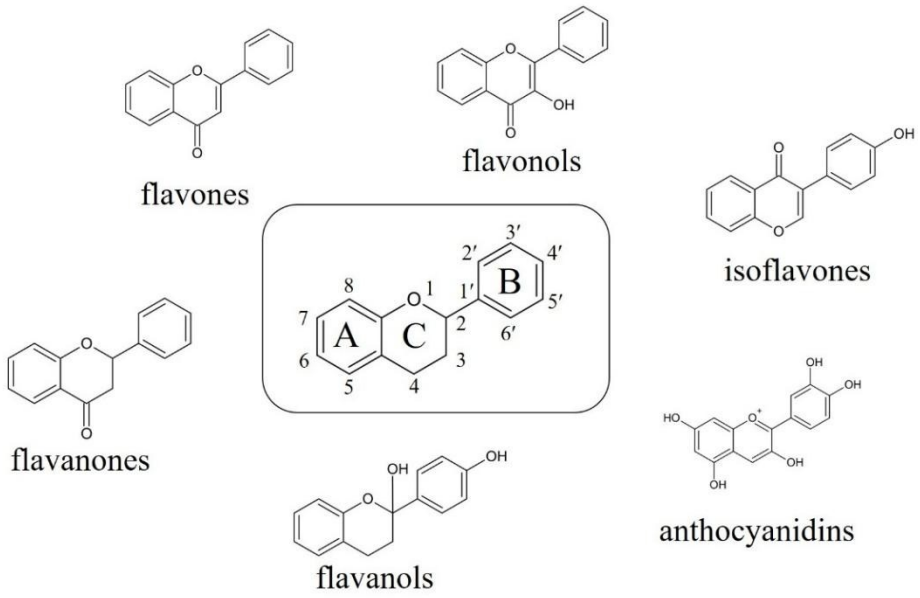


Figure 1. *Schematic representation of the core flavonoid structure and its six major subclasses (own illustration).*

Like other phenolic compounds, flavonoids are synthesized in plants via two biosynthetic routes: the phenylpropanoid pathway, which generates the C6-C3 backbone, and the polyketide pathway, which provides the C2 building units. These routes converge with *p*-coumaroyl-CoA and malonyl-CoA as initial substrates, which are converted by the enzyme chalcone synthase into the 2′-hydroxychalcone scaffold (Dias et al., 2021). The expression of genes involved in flavonoid biosynthesis is modulated by numerous environmental factors, including light, temperature, hormones, and various stress stimuli (Patil et al., 2024). Thus, flavonoids are distributed across diverse plant tissues, including leaves, flowers, seeds, and fruits. Depending on their biosynthetic origin, they fulfil a variety of ecological and physiological functions, ranging from pigmentation, seed development, allelopathy, and pollination, to protective roles against abiotic stressors such as UV-B radiation, extreme temperatures, heavy metals, salinity, and drought (Zhuang et al., 2023; Šamec et al., 2021). Although flavonoids play a crucial role in plant growth and development, they have attracted substantial scientific interest because of their diverse biological activities in humans and their potential health-promoting effects, which have been extensively investigated over the past thirty years (Alfa & Arroo, 2019).

The type and position of substituents largely determine the biological role and activity of flavonoids. For instance, both the arrangement of hydroxyl groups in the catechol B-ring and their orientation relative to the C-ring strongly influence the antioxidant capacity of flavonoids

(D'Amelia et al., 2018). An integrated multi-omics approach further demonstrated that the bioactivity of *Ormosia henryi* flavonoids is strongly conditioned by their structural diversity, with hydroxylated and methoxylated derivatives exhibiting enhanced antioxidant, enzyme-inhibitory, and antibacterial activities through interactions with inflammation- and metabolism-related molecular targets (Zhang et al., 2025). An overview of representative structure-activity relationships and their putative roles in plants is summarized in Table 1.

***Table 1**. Overview of flavonoid structural features in relation to biological activity (health-related effects) and roles in plants.*

Structural feature	Structure - biological activity relationship	Mechanism	References
Prenylation	Enhances antioxidant, anti-inflammatory, anticancer, and metabolic regulatory activities	Modulation of key signaling pathways (NF-κB, STAT3, Nrf2); inhibition of tumor growth, angiogenesis, and metastasis; regulation of glucose and lipid metabolism; interaction with cellular membranes and protein targets facilitated by increased lipophilicity	Altalbawy et al., 2026
Hydroxylation and site-specific *O*-methylation pattern	Selective *O*-methylation of quercetin (notably at C-3 and C-4′) enhances antiproliferative activity against colorectal cancer cells while preserving low toxicity toward normal cells	Induction of cell cycle arrest and apoptosis mediated by oxidative stress, mitochondrial dysfunction, and inhibition of the SRC/JAK2/STAT3 signaling pathway	Han et al., 2025
Prenylation	Enhances antibacterial activity, particularly against *Staphylococcus aureus* and MRSA; increased number of prenyl groups correlates with stronger activity	Inhibition of nucleic acid synthesis disruption of cytoplasmic membrane function; interference with energy metabolism, including ATP synthase inhibition by common flavonoids	Herlina et al., 2025
7-*O*-glycodylation with additional acylation	Enhances α-glucosidase inhibition	Increased hydrogen bonding and hydrophobic interactions with α-glucosidase, leading to	Chen et al., 2025

		enzyme conformational changes and reduced activity	
Acylated flavonoid glycoside	Anti-inflammatory activity	Inhibition of nitric oxide (NO) production in LPS-stimulated RAW264.7 macrophages	Vien et al., 2023
Prenylation	Anticancer activity	Induction of apoptosis, inhibition of angiogenesis, and modulation of key regulatory targets, including epigenetic factors, transcription factors, COX-2, and kinases	Summarized by Wen et al., 2022
O-methylation	Determine membrane interaction behavior, localization within lipid bilayers, and protein-binding properties without inducing cytotoxicity	Non-hemolytic interaction with red blood cell and model membranes; modulation of membrane packing and fluidity in hydrophilic or hydrophobic regions depending on scaffold type; spontaneous binding to human serum albumin via hydrogen bonding and van der Waals interactions (static quenching)	Włoch et al., 2021

Structural feature	**Structure – role in plants**	**Mechanism**	**References**
Hydroxylation pattern	Hydroxylation critically determines flavonoid functions in plants, including photoprotection, antioxidant defense, pigmentation, allelopathy, pathogen resistance, and regulation of plant–microbe and plant–plant interactions	Enhanced ROS scavenging and metal chelation, particularly in dihydroxylated B-ring flavonoids (e.g. catechol-type structures); UV protection mediated by dihydroxy B-ring flavonoids (e.g. quercetin and luteolin derivatives); modulation of auxin transport and signaling; contribution to pigmentation intensity and color diversification (increased B-ring hydroxylation enhances blue coloration); involvement in defense against herbivores, pathogens, and competing plants (allelopathy)	Summarized by Alseekh et al., 2020

Glycosylation (*O*- and *C*-glycosylation)	Modulates flavonoid function by enhancing solubility, stability, transport, and storage, thereby influencing pigmentation, stress tolerance, defense responses, taste, and species or cultivar-specific traits	Increased molecular solubility and chemical stability of flavonoids; essential for anthocyanin stabilization, vacuolar transport, and color diversification; contribution to resistance against pathogens and herbivores (e.g. C-glycosyl flavonoids in rice blast resistance); regulation of fruit quality and taste (e.g. bitterness in citrus via flavanone glycosides); involvement in early fruit development and protection (proanthocyanidin glycoside modification)	Summarized by Alseekh et al., 2020

Given that plants are sessile organisms, flavonoids represent a crucial component of plant defense under stress conditions, contributing to tolerance against abiotic stresses by enhancing antioxidant capacity, regulating cellular redox homeostasis, and participating in stress-response signaling networks. Their biosynthesis and accumulation are dynamically induced by environmental cues, enabling flavonoids to modulate physiological processes at both biochemical and cellular levels, including stomatal function, membrane stability, and transcriptional regulation (Shomali et al., 2022).

Accordingly, flavonoids have been the focus of sustained scientific interest for decades as promising candidates in drug design and disease prevention. Flavonoids exert a broad spectrum of therapeutic effects, including antioxidant, anti-inflammatory, antimicrobial, antiviral, anticancer, cardioprotective, metabolic, and neuroprotective activities, underscoring their pleiotropic mode of action and relevance in complex disease states (summarized by Stachelska et al. (2025)). Representative flavonoid compounds, their health-related effects, and the underlying mechanisms of action are summarized in Table 2.

Table 2. *Overview of representative flavonoid compounds and their reported health-related effects, together with the underlying mechanisms of action.*

Flavonoid compounds	Health-related effect	Underlying mechanism of action	References
Luteolin	Neuroprotection in Huntington's disease; increased neuronal viability; reduction of mutant huntingtin toxicity	Attenuation of mutant huntingtin–induced cytotoxicity by reducing both soluble and insoluble htt aggregates; suppression of apoptosis via decreased caspase-3 activation; neuroprotective effect independent of the Nrf2/HO-1 antioxidant pathway	Ramadan et al., 2023
Rutin	Neuroprotection in acute ischemic stroke-reperfusion injury; reduction of neurological damage; enhancement of neuroplasticity and angiogenesis in the ischemic penumbra	High affinity binding to ACE2 receptors and activation of the ACE2/Ang-(1–7) signaling pathway; attenuation of neuroinflammation; promotion of angiogenesis and neovascularization; mitochondrial protection enhanced by BBB-penetrating, CD44-targeted, and pH/hyaluronidase-responsive nanodelivery system	Zhao et al., 2023
Kaempferol	Neuroprotection in Parkinson's disease; improvement of motor behavior; protection of dopaminergic neurons	Inhibition of microglial pyroptosis and neuroinflammation via downregulation of NLRP3 inflammasome components (NLRP3, ASC, caspase-1, GSDMD-NT); suppression of pro-inflammatory cytokines (IL-1β, IL-18), iNOS and COX-2; inhibition of the p38MAPK/NF-κB signaling pathway *in vivo* (6-OHDA PD rats) and *in vitro* (LPS-stimulated BV2 cells)	Cai et al., 2022
Acacetin	Neuroprotective; improvement of learning and memory; attenuation	Inhibition of the NLRP3 inflammasome signaling pathway; downregulation of NLRP3, caspase-1, IL-1β and	Bu et al., 2022

	of Alzheimer's disease pathology	TNF-α; reduction of amyloid-β42 levels and senile plaque formation	
Baicalein	Neuroprotective effect in Parkinson's disease; protection of dopaminergic neurons; improvement of motor and neurological deficits	Activation of mitochondrial autophagy (mitophagy) via downregulation of miR-30b-5p and upregulation of SIRT1; modulation of the SIRT1/AMPK/mTOR signaling pathway; restoration of mitochondrial function and reduction of neuronal apoptosis	Chen et al., 2022
Various flavonoids (66 reported)	Antibacterial activity against Gram-positive bacteria	Antibacterial potency strongly correlated with lipophilicity; primary target is the bacterial cell membrane, involving disruption of phospholipid bilayers, inhibition of respiratory chain and/or ATP synthesis	Yuan et al., 2021
Vitexin	Antiviral activity against influenza A (H1N1); anti-inflammatory and immunomodulatory effects	Dynamic modulation of innate immune response via TLR signaling pathways: partial down-regulation of TLR3 and TLR7 pathways and up-regulation of TLR4 signaling; suppression of pro-inflammatory mediators (NO, IL-6, TNF-α) and enhancement of IFN-β production, promoting viral clearance	Shi et al., 2020
Quercetin	Anticancer activity; anti-inflammatory and antioxidant effects	Modulation of cell cycle progression, inhibition of cell proliferation, induction of apoptosis, suppression of angiogenesis and metastasis, regulation of autophagy	Tang et al., 2020
Apigenin	Anti-inflammatory and neuroprotective effects in chronic diseases (cancer, diabetes, cardiovascular and neurodegenerative disorders)	Suppression of chronic low-grade inflammation; modulation of inflammatory signaling pathways; reduction of immune cell infiltration and edema in CNS; protection against tissue injury and demyelination; low toxicity and non-mutagenic profile	Ginwala et al., 2019

Flavonoids are key dietary phytochemicals, linking high fruit and vegetable consumption with improved human health and supporting their growing use as nutraceuticals (Safe et al., 2021). Accordingly, flavonoids such as quercetin have gained considerable commercial relevance and are increasingly incorporated into functional foods and nutraceutical formulations (Tang et al., 2020). This general health-promoting role is further substantiated by large-scale, long-term epidemiological studies. Specifically, more than two decades of dietary follow-up within the Nurses' Health Study, together with a 14-year follow-up of health professionals in the Health Professionals Follow-up Study (Bondonno et al., 2025), have provided valuable insights into the relationship between flavonoid-rich dietary intake and aging-related outcomes. The findings demonstrated that participants with the highest flavodiet scores exhibited up to a 15% lower risk of frailty, approximately a 12% reduced risk of impaired physical function, and about a 12% lower risk of poor mental health. Overall, these results indicate that a diet rich in flavonoids may substantially contribute to the preservation of functional and mental health and promote healthy aging.

However, regarding their impact on human health, it is important to note that most flavonoids exhibit low bioavailability. Absorption is limited and depends on the subclass, while extensive modifications by gastrointestinal microbiota, chemical transformations, and rapid excretion further reduce systemic availability. Only aglycones and some glucosides can be absorbed in the small intestine (Tsanova-Savova et al., 2018). Flavonoid glycosides require prior deglycosylation, a process that depends on the structure and position of the sugar moiety (Gonzales et al., 2015). Consequently, numerous studies (summarized by Li et al. (2023)) have focused on structural modification of flavonoids to improve their bioavailability and biological efficacy, employing chemical, enzymatic, and biotechnological approaches such as methylation, glycosylation, prenylation, and acylation.

1.2. Plant biflavonoids: structural classes, ecological roles, and advances in dimerization coupling

As mentioned above, one of the structural modifications of flavonoids that occurs in plants is dimerization - a process in which two flavonoid monomers are covalently linked to form biflavonoids. These dimers consist of two identical or different monomeric units that may be connected symmetrically or asymmetrically through alkyl or alkoxy bridges of varying length (Šamec et al., 2022). To date, nearly 600 biflavonoids have been identified, distributed across almost all terrestrial plant groups, with the highest occurrence in angiosperms (He et al., 2021).

However, their exact distribution has not been clearly established, as biflavonoids have mostly been identified sporadically, and there are relatively few scientific studies focused exclusively on them (summarized by Šamec et al. (2022)). Based on the type of monomeric units involved, 17 structural categories are distinguished, the most common being flavone–flavone, flavone–flavanone, and flavanone–flavanone combinations (He et al., 2021). Each of these groups further differs in the position of the carbon atom through which the monomers are connected. Most of the available literature reports biflavonoids as aglycone molecules, but there are also examples where they occur as glycosylated forms, such as in the whisk fern (*Psilotum nudum* L.) (Šamec et al., 2019).

Despite being known for nearly a century, biflavonoids have recently attracted renewed scientific interest due to their broad spectrum of bioactivities, predominantly explored through *in vitro* experiments and *in silico* analyses, while *in vivo* evidence remains comparatively limited. These investigations highlight their antioxidant, anti-inflammatory, antiviral, antimicrobial, antifungal, and antitumor effects, collectively positioning biflavonoids as promising agents for the prevention and treatment of various pathological conditions (summarized in Table 3).

Table 3. *Overview of biological activities of biflavonoids reported in the literature.*

Tested biflavonoid	Method	Activity	References
amentoflavone	ML-based network pharmacology; docking and MD; *in vitro* migration assays; *in vivo* metastasis model	Antimetastatic; inhibits NSCLC cell migration and EMT via TGF-β/Smad pathway	Liu et al., 2026
(2S,3S)-Volkensiflavone-7-*O*-β-glucopyranoside; (2R,3S)-Volkensiflavone-7-*O*-β-D-acetylglucopyranoside	*In vitro* fluorimetric enzyme inhibition assays (MAO-A, BACE-1, GSK-3β); inhibition kinetics; *in silico* molecular docking	Anti-Alzheimer potential; triple inhibition of MAO-A, BACE-1, and GSK-3β (multi-target-directed ligands)	Ella et al., 2026
amentoflavone, robustaflavone, cupressuflavone	*In silico* molecular docking	Antioxidant (CA12/CA9 binding; antidiabetic (α-glucosidase, α-amylase inhibition); anti-inflammatory (COX-2/LOX-5 inhibition)	Khanna et al., 2025

hinokiflavone	*In silico* target prediction, docking and MD; *in vitro* RCCED model; *in vivo* HFD-induced ED rat model	Improves erectile function; antioxidant and endothelial protective effects via EGFR/Akt signalling	Gao et al., 2025
kolaviron	*In vivo* DSS colitis model; *in silico* docking	Ameliorates ulcerative colitis; antioxidant and anti-inflammatory; increases MUC-2 expression	Adeniran et al., 2025
amentoflavone, putraflavone, podocarpusflavone A	*In vitro* LPS-stimulated RAW264.7 macrophage model; PGE_2 and NO assays; mRNA expression analysis (TNF-α, COX-2, iNOS, NF-κB)	Anti-inflammatory; inhibits PGE_2 and NO production; downregulates TNF-α, COX-2, and iNOS (amentoflavone most potent)	Mangmool et al., 2024
strychnobiflavone	*In silico* docking-based virtual screening; molecular dynamics simulations; *in vitro* enzyme kinetics	Antidiabetic α-glucosidase inhibition, induces conformational changes affecting catalytic activity	Sadeghi et al., 2023
amentoflavone, hinokiflavone	*In vitro* α-glucosidase inhibition assays; multispectral analysis; *in silico* molecular docking	Antidiabetic potential; strong noncompetitive α-glucosidase inhibition; synergistic effect with acarbose; induces conformational changes of the enzyme	H. Li et al., 2023
(R)-rhizomatobiflavonoid A, (R)-rhizomatobiflavonoid B, (R)-rhizomatobiflavonoid C	*In vitro* enzyme inhibition (HIV-1 integrase; antiplasmodial assay); *in silico* molecular docking	Anti-HIV (HIV-1 integrase inhibition); antimalarial	Messi et al., 2022
selaginellin	*In vitro* melanocyte assays; *in vivo* pigmentation model; signaling pathway analysis (MAPK/MITF/TYR/TYRP2)	Antimelanogenic; inhibits melanogenesis via MAPK pathway; downregulates MITF, TYR, and TYRP2; antagonizes UVB-induced paracrine signaling	Zhou et al., 2022

luteolin-(6-8″)-apigenin	*In vitro* α-amylase inhibition assay; erythrocyte membrane stabilization test	Antidiabetic (α-amylase inhibition); anti-inflammatory (membrane-stabilizing effect)	El-Nashar et al., 2022
dysosmabiflavonoids (A-G)	*In vitro* AChE inhibition assay; NO inhibition in LPS-stimulated RAW264.7 cells; mushroom tyrosinase inhibition assay; SAR analysis	Potent AChE inhibition, anti-inflammatory potential, tyrosinase inhibition; potential for neurodegenerative and pigmentation disorders	Sun et al., 2022
hinokiflavone	Review of *in vitro/in vivo* studies; molecular docking; formulation studies	Anticancer; antiproliferative and antimetastatic; modulates ERK1/2-p38-NFκB signaling, downregulates MMP-2/MMP-9; SENP1 inhibition (pre-mRNA splicing)	Goossens et al., 2021
amentoflavone, bilobetin, podocarpusflavone, sequoiaflavone, sotetsuflavone	Thioflavin T (ThT) fluorescence assay; *in silico* molecular docking; molecular dynamics simulations	Anti-amyloidogenic; promotes disaggregation of Aβ fibrils; reduces β-sheet content via π–π interactions and hydrogen bonding	Windsor et al., 2021
5,7,7″,4‴-tetra-*O*-methyl-hinokiflavone, hinokiflavone, 2,3-dihydrobilobetin, 4′,4‴-*O*-dimethyl-amentoflavone	*In vitro* antibacterial assays against *Klebsiella pneumoniae* (MIC determination); membrane integrity and permeability assays; SEM; efflux gene expression (qRT-PCR)	Strong antibacterial activity (MIC 0.25-2 μg/mL); disrupts membrane integrity and permeability; reduces efflux activity	Negm et al., 2021
sciadopitysin	*In vitro* SARS-CoV-2 3CLpro inhibition assay; inhibition kinetics*; in silico* molecular docking	Antiviral; potent SARS-CoV-2 3CLpro inhibition (IC_{50} < 2 μM); reversible mixed-type inhibition	Xiong et al., 2021
rhusflavanone, mesuaferrone B	*In vitro* DPPH radical scavenging assay; human leukocyte elastase inhibition assay; mushroom tyrosinase inhibition assay	Strong elastase and tyrosinase inhibition (cosmetic/anti-aging potential); low antioxidant activity	Zar Wynn Myint et al., 2021
selamariscina A, amentoflavone, robustaflavone, cupressuflavone, taiwaniaflavone	*In vitro* CYP450 inhibition in human liver microsomes (cocktail incubation); LC–MS/MS; UGT inhibition assays	Selective inhibition of CYP2C8 and CYP2C9 (noncompetitive/competitive); potential herb–drug interaction risk; weak UGT inhibition	Park et al., 2020

agathisflavone	*In vitro* neuraminidase inhibition assays (wild-type and OST-resistant influenza strains); viral replication assays; resistance mutation analysis	Antiviral; inhibits influenza neuraminidase and viral replication; active against oseltamivir-resistant strains via alternative NA binding site	de Freitas et al., 2020
bilobetin, isoginkgetin	*In vitro* antiproliferative assays on cancer cell lines; cell cycle analysis; apoptosis assays; protein expression analysis (Bax, Bcl-2, Caspase-3)	Anticancer; inhibits cell proliferation; G2/M cell cycle arrest; induces apoptosis via Bax and caspase-3 activation (bilobetin also downregulates Bcl-2)	M. Li et al., 2019
amentoflavone	*In vitro* antioxidant assays (DPPH, ABTS, superoxide, hydroxyl radicals); antibacterial assays (MIC); membrane depolarization and permeability assays; SEM/TEM; food model systems (minced chicken, apple juice)	Strong antioxidant activity; antibacterial against *S. aureus* and *E. coli* via membrane disruption; effective in food preservation models	Bajpai et al., 2019
amentoflavone, agatisflavone, sequoiaflavone, putraflavone, podocarpusflavone, 7,7″-*O*-methyllanaraflavone	*In vivo* CFA-induced arthritis rat model; *in vitro* oxidative stress and cell viability assays	Anti-inflammatory and antinociceptive; reduces cytokines (IL-1β, IL-6, TNF-α), improves joint function, decreases bone resorption; antioxidant activity	Oliveira et al., 2019
amentoflavone, sciadopitysin, ginkgetin, isoginkgetin, bilobetin	*In vitro* cytotoxicity assays (HK-2, L-02 cells); *in vivo* mouse model (intragastric administration, serum biochemistry, histopathology, TUNEL assay)	Potential hepatotoxicity and nephrotoxicity; reduced cell viability; induces acute kidney injury via apoptosis (↑BAX, unchanged BCL-2)	Li et al., 2019

Among the various C-C-linked biflavonoid subtypes, 3′,8″-biflavonoids form one of the most prominent groups. An overview of selected plant species known to contain these dimers, ranging from ferns to angiosperms, along with their representative compounds, is provided in Table 4. Many of these plant species have been recognized in traditional medicine and, more recently, in pharmacological research due to their reported health-promoting effects and therapeutic potential (Figure 2).

Table 4. *Overview of selected plant species and their common 3′,8″-biflavonoids (adapted from Šamec et al., 2024).*

Plant species		Typical 3′,8″-biflavonoids
Latin name	Common name	
Psilotum nudum (L.) Beauv.	Whisk fern, Skeleton fork fen	amentoflavone
Selaginella denticulata (L.) Spring	Mediterranean clubmoss	amentoflavone sotetsuflavone
Cupressus sempervirens L.	Mediterranean cypress, Common cypress	amentoflavone methylamentoflavone 2,3-dihydroamentoflavone
Cycas revoluta Thunb.	Japanese cycad, Sago palm	amentoflavone podocarpusflavone A (2S)-2,3-dihydroamentoflavone (2S,2″S)-2,3,2″,3″-tetrahydroamentoflavone
Ginkgo biloba L.	Ginko, Maidenhair tree	amentoflavone bilobetin ginkgetin isoginkgetin sciadopytysin 5'-methoxybilobetin
Juniperus occidentalis L.	Western juniper, Sierra juniper	amentoflavone
Metasequoia glyptostroboides Hu and Cheng	Dawn redwood, Water larch	sequoiaflavone podocarpusflavone A podocarpusflavone B isoginkgetin sciadopitysin amentoflavone

		2,3-dihydroamentoflavone-7″,4‴-dimethyl ether amentoflavone-7″,4‴-dimethyl ether bilobetin ginkgetin 2,3-dihydroisoginkgetin 2,3-dihydrosciadopitysin ginkgetin sciadopitysin
Taxus baccata L.	Common yew, English yew	amentoflavone bilobetin podocarpusflavone A sequoiaflavone
Hypericum perforatum L.	St. John's wort	amentoflavone

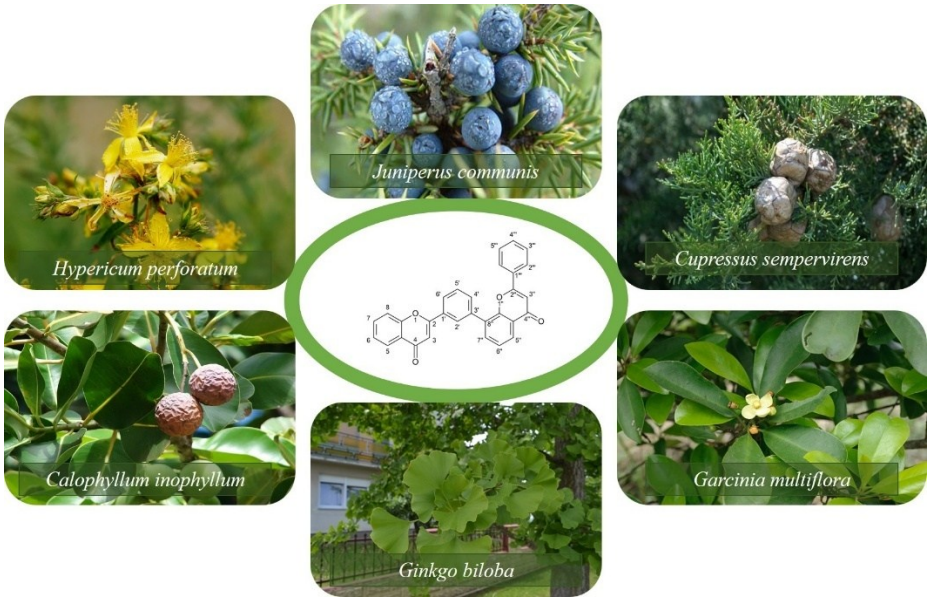


***Figure 2**. Selected medicinal plant species reported to produce 3',8''-biflavones (own illustration).*

Although several studies have described the chemically induced synthesis of biflavonoids (Zheng et al., 2025; Sum et al., 2018), their natural biosynthetic pathway in plants remained largely unknown until recently. This gap was resolved by Dai et al. (2025), who identified gymnosperm-specific cytochrome P450 enzymes of the CYP90J subfamily as the missing link in biflavonoid biosynthesis. These enzymes catalyze highly regioselective intermolecular C-C dimerization of flavone monomers and, together with specific *O*-methyltransferases, enable the formation of naturally occurring ginkgo biflavonoids. While intramolecular C-C and C-O phenolic coupling reactions had been reported previously (An et al., 2024; Meng et al., 2024), the intermolecular C-C coupling of flavonoids - an essential step in the biosynthesis of most natural biflavonoids - had remained largely unexplored. Recent advances have now provided the first mechanistic insight into the biosynthesis of major natural dimers, including 3′,8″-biflavones, one of the most abundant and biologically active classes of C-C-linked biflavonoids. In gymnosperms, CYP90J enzymes act as highly selective catalysts of oxidative C-C dimerization between two flavone units in a defined 3′,8″ orientation (Figure 3) (Dai et al., 2025).

These enzymes operate through a diradical mechanism, in which steric constraints within the active site, interactions with surrounding amino acid residues, and π–π stabilization between the two flavonoid substrates orient the monomers to enable regioselective formation of the 3′,8″ linkage. This discovery represents a major step forward in elucidating biflavonoid biosynthesis and establishes a foundation for further exploration of their functional roles in plants, as well as for developing biotechnological platforms for the production of high-value 3′,8″-biflavones (Dai et al., 2025).

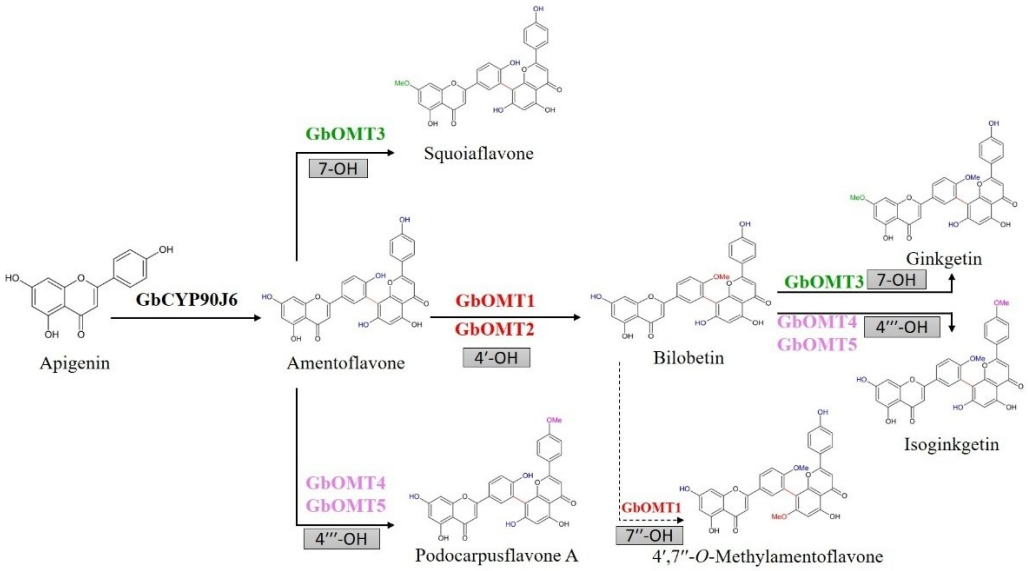


Figure 3. *Biosynthetic pathway of amentoflavone and its methylated derivatives in ginkgo. GbCYP90J6 catalyzes the dimerization of apigenin to form amentoflavone, which serves as a common precursor for bilobetin, ginkgetin, isoginkgetin, podocarpusflavone A, sequoiaflavone, and 4',7''-O-methylamentoflavone. GbOMT1 and GbOMT2 catalyze the conversion of amentoflavone to bilobetin, which is further converted to ginkgetin by GbOMT3 or to isoginkgetin by GbOMT4 and GbOMT5. Podocarpusflavone A, sequoiaflavone, 4',7''-O-methylamentoflavone represent shunt products (adapted from Dai et al., 2025).*

Given that dimerization represents a key structural factor influencing the biological activity of biflavonoids, this doctoral dissertation provides the first comprehensive evaluation of the *in vitro* biological activities of purified 3′,8″-biflavone standards in direct comparison with their corresponding monomeric flavonoids (Article 1). All compounds were systematically assessed for antioxidant activity using the DPPH radical scavenging assay, antifungal potential through minimum inhibitory concentration (MIC) testing against major mycotoxigenic fungi relevant to food safety and public health, and their inhibitory effects on enzymes of clinical relevance. This included acetylcholinesterase, tyrosinase, and the carbohydrate-hydrolyzing enzymes α-glucosidase and α-amylase, which play key roles in neurodegenerative disorders, pigmentation processes, and glucose metabolism, respectively.

1.3. Ginkgo - a model plant for the study of 3′,8″-biflavones

The first natural biflavonoid to be isolated was ginkgetin, obtained nearly a century ago from the yellow leaves of ginkgo (*Ginkgo biloba* L.). To this day, ginkgo remains one of the best-known sources of biflavonoids. Ginkgo is the only surviving representative of the division Ginkgophyta. As an ancient lineage - with fossil records tracing back to the Mesozoic Era (Early Jurassic, ~180 million years ago) - ginkgo is considered a "living fossil" due to its distinctive morphological features, including fan-shaped leaves, dichotomous venation and branching (Figure 4), as well as the presence of motile, multi-flagellated sperm cells. Originating in China, ginkgo began spreading across the world approximately 300 years ago through human-mediated cultivation (Stanković, 2016). Today, it is a highly valued species in landscape and urban architecture, appreciated for its unique appearance, its striking autumnal leaf color transition to bright yellow (Figure 4), and its remarkable tolerance to pollution, pests, and harsh urban environments (Guo et al., 2023).

Ginkgo is dioecious, producing separate male and female trees, with pollination occurring via wind. Female trees bear elliptic seeds enclosed in a fleshy outer layer (sarcotesta), which is soft, yellow-brown, and coated with a whitish bloom. Beneath it lies a hard sclerotesta that protects the kernel (endosperm), a traditional ingredient in Chinese cuisine (Figure 4). Due to the presence of butyric acid, the sarcotesta emits a characteristic strong and unpleasant odor, making female trees generally undesirable in horticulture (Guo et al., 2023). The extraordinary longevity and environmental resilience of ginkgo - including survival of extreme events such as the Hiroshima bombing - are attributed in part to its rich and complex phytochemical profile.

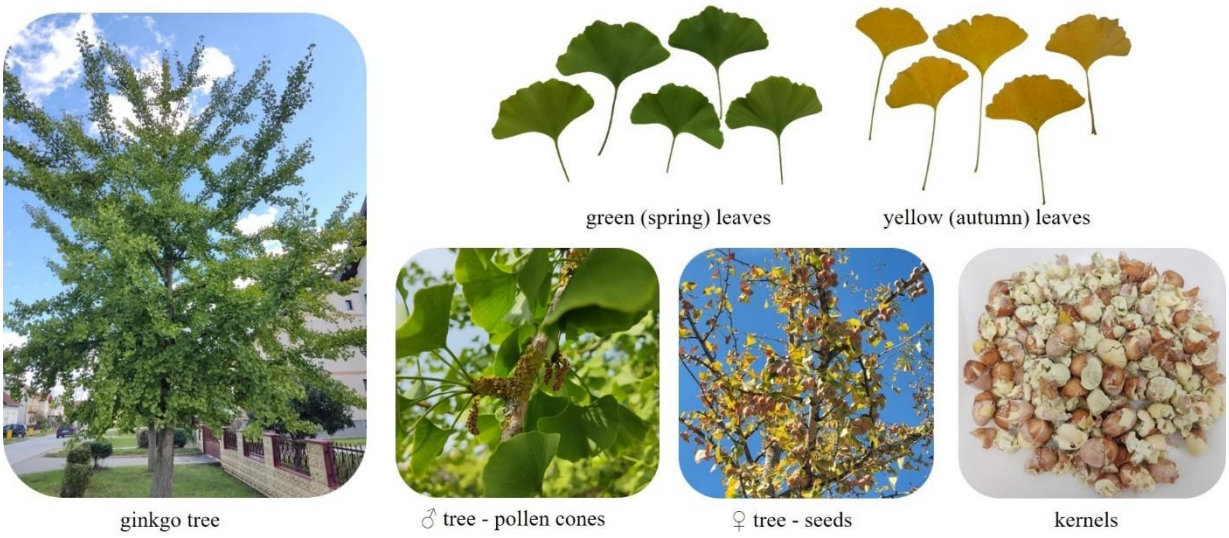


Figure 4. *Main morphological characteristics of ginkgo (own illustration).*

Ginkgo has a long history of use in traditional Chinese medicine, where various plant parts were employed for a wide range of ailments, including respiratory problems such as bronchitis and asthma, gastrointestinal discomfort, and even tuberculosis. Leaves were valued for detoxifying properties and for addressing digestive and urinary disorders. Over time, interest in ginkgo expanded due to its influence on cognitive function (Akaberi et al., 2023). Numerous clinical studies have since confirmed its efficacy as both a dietary supplement and a medicinal preparation for memory enhancement (Ge et al., 2021; Singh et al., 2019). Moreover, ginkgo extracts show promise in the prevention and management of Alzheimer's disease and other neurodegenerative conditions. Owing to their immunomodulatory, anti-inflammatory, neuroprotective, and antioxidant effects, ginkgo preparations are increasingly explored as therapeutic candidates for cardiovascular disorders as well (Akaberi et al., 2023).

1.3.1. Phytochemicals in ginkgo

Ginkgo contains a chemically diverse array of phytochemicals that can be classified into several major groups, including flavonoids, terpenoids, carboxylic acids, lignins, proanthocyanidins, polyprenols, polysaccharides, alkyphenols, and alkyphenolic acids (Biernacka et al., 2023). These compounds differ in structure, abundance, and biological relevance, and their relative proportions may vary depending on the plant material and extraction conditions (Kulić et al., 2022).

To date, 110 flavonoids have been reported in ginkgo (summarized by Liu et al. (2021)). These flavonoids can be classified into several major groups, among which flavonols and their glycosides represent the most abundant. Among these, derivatives of quercetin, kaempferol, and isorhamnetin predominate, while myricetin, syringetin, laricitrin, and patuletin are less frequently represented. Another important group consists of flavones and flavone glycosides, with apigenin and luteolin as the principal representatives. In addition, flavanones, isoflavones, flavan-3-ols, and a considerable number of biflavonoids were detected. A distinct subgroup comprises biginkgosides, more recently identified compounds that are classified as flavonol glycosides (Liu et al., 2021).

Flavonoids, together with their glycosides, form the basis of the biological activity of ginkgo, owing to their broad range of effects, including antioxidant, neuroprotective, anti-inflammatory, antimicrobial, and other beneficial actions on human health (Biernacka et al., 2023; Šamec et al., 2022). Some of the most frequently reported flavonoids are shown in Figure 5.

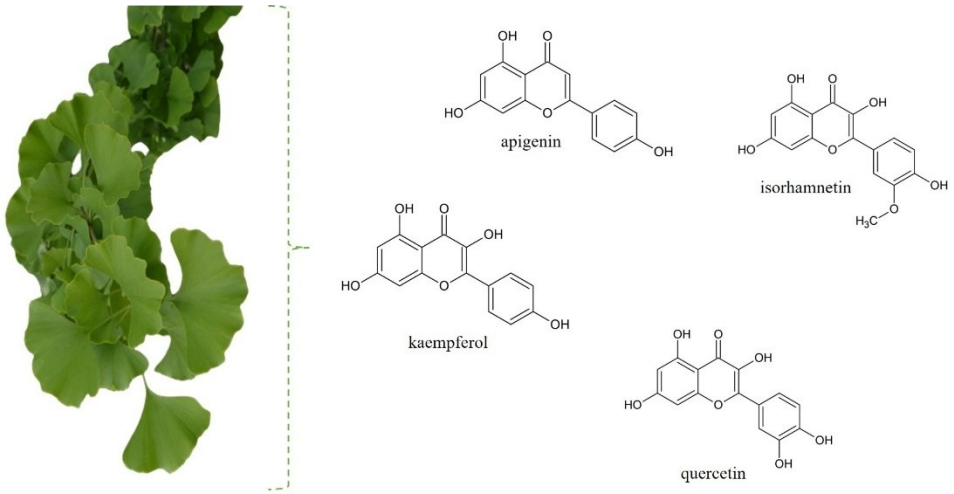


***Figure 5**. Selected examples of frequently reported flavonoids (own illustration).*

Phytochemically, the richest part of the plant is its foliage, which contains more than 180 identified compounds, including flavonoids, terpenoids, and various polyphenols (Guo et al., 2023). The standardized ginkgo dry extract is obtained from the leaves, and is adjusted to 22-27% flavone glycosides, 2.6-3.2% terpene lactones (bilobalide), and 2.8-3.4% ginkgolides A, B, and C, while the total content of ginkgolic acids must not exceed 5 ppm (European Pharmacopoeia, 2019). Annual global production of ginkgo extract exceeds 3 million tons, accounting for roughly one-sixth of worldwide demand. Among flavonoids, kaempferol, quercetin, and isorhamnetin are the most abundant (Guo et al., 2023).

1.3.2. Biflavonoids in ginkgo

To date, nearly 600 biflavonoids have been reported in plants (He et al., 2021). Among them, based on the literature, ginkgo synthesizes exclusively 3′,8″-linked biflavonoids, named according to the carbon atoms through which the two monomeric units are joined (Figure 6a). The first such dimer to be isolated was ginkgetin, which was obtained as a yellow pigment from the yellow autumn leaves of ginkgo (Figure 6b) (Šamec et al., 2023). Subsequently, other biflavonoids were isolated from ginkgo, and in addition to ginkgetin, the compounds isoginkgetin and bilobetin were also named after ginkgo.

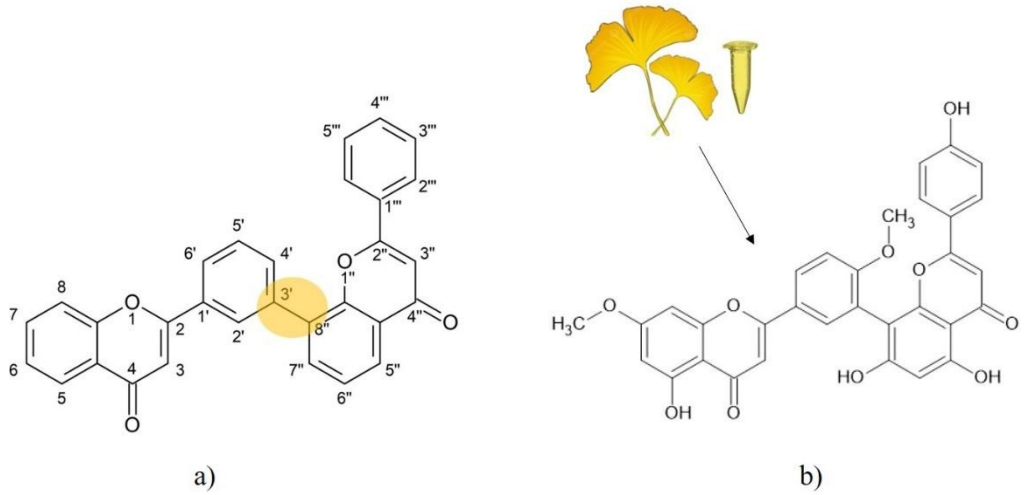
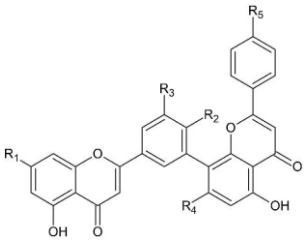


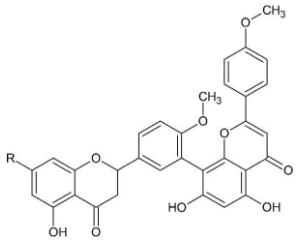
Figure 6. *Basic chemical structure of 3',8''-biflavones (a) and the first isolated biflavonoid, ginkgetin (b) (own illustration).*

To date, 13 biflavonoids have been reported in ginkgo (L. Liu et al., 2021) (Table 5), with amentoflavone, ginkgetin, isoginkgetin, bilobetin, and sciadopitysin being the most common representatives (Figure 7) (Šamec et al., 2024, 2022).

Table 5. *Chemical structure of 13 biflavonoids in ginkgo (adapted from Šamec et al., 2022).*



	R_1	R_2	R_3	R_4	R_5
amentoflavone	OH	OH	H	OH	OH
sequoiaflavone	OCH_3	OH	H	OH	OH
bilobetin	OH	OCH_3	H	OH	OH
podocarpusflavone A	OH	OH	H	OH	OCH_3
ginkgetin	OCH_3	OCH_3	H	OH	OH
isoginkgetin	OH	OCH_3	H	OH	OCH_3
5′-methoxybilobetin	OH	OCH_3	OCH_3	OH	OH
sciadopitiysin	OCH_3	OCH_3	H	OH	OCH_3
7′′-*O*-β-D-glucosyl-ginkgetin	OCH_3	OCH_3	H	O-Glc	OH
7′-*O*-β-D-glucosyl-isoginkgetin	O-Glc	OCH_3	H	H	OCH_3
amentoflavone 7′′-*O*-D-glucopyranoside	OH	OH	H	O-Glc	OH



	R
2,3-dihydroisoginkgetin	OH
2,3-dihydrosciadopitysin	OCH_3

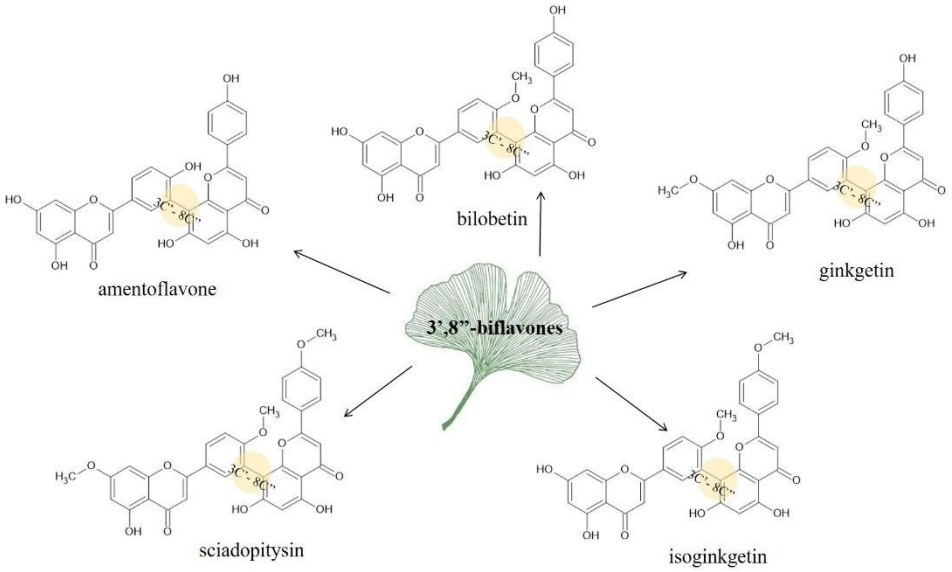
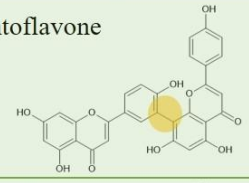
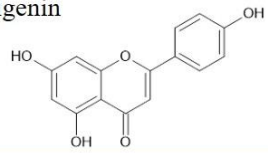
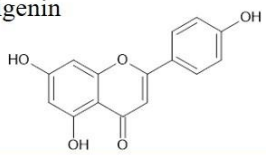
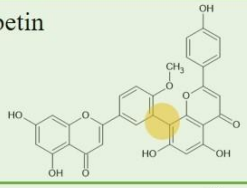
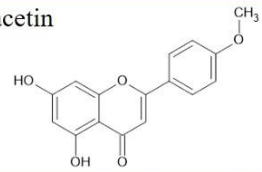
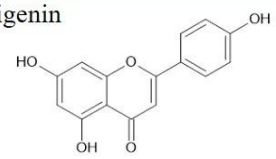
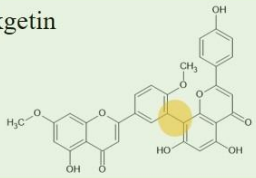
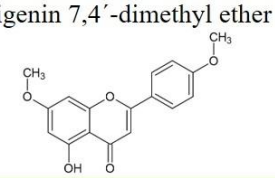
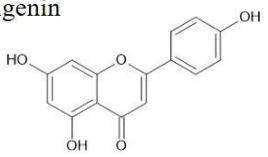
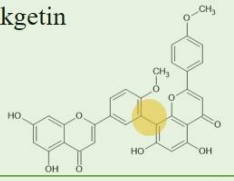
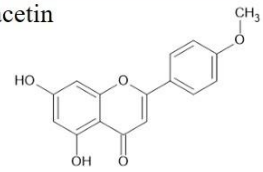
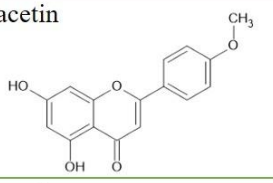
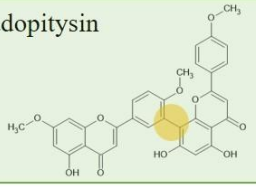
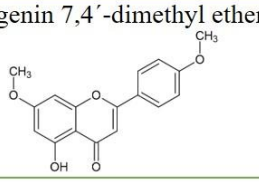
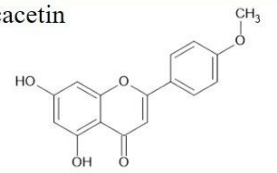


Figure 7. *The structure of the five most prevalent 3',8''-biflavones in ginkgo (own illustration).*

Although all 3′,8″-biflavones share the same characteristic C-C linkage, their structural diversity arises from variations in the constituent monomeric flavonoids and in the type and position of substituents, including methyl and hydroxyl groups (Table 6). Amentoflavone is a dimer of two apigenin molecules containing six hydroxyl groups that can be readily methylated; consequently, the remaining major biflavonoids found in ginkgo are commonly regarded as structural derivatives of amentoflavone (Šamec et al., 2022).

Table 6. *Structure of 3′,8″-biflavones and their monomeric flavonoid units.*

3′,8″-biflavones	monomers	
amentoflavone 	apigenin 	apigenin 
bilobetin 	acacetin 	apigenin 
ginkgetin 	apigenin 7,4′-dimethyl ether 	apigenin 
isoginkgetin 	acacetin 	acacetin 
sciadopitysin 	apigenin 7,4′-dimethyl ether 	acacetin 

As is evident from Table 5 in ginkgo, several naturally occurring mono-*O*-methylated derivatives have been identified, including sequoiaflavone, bilobetin, and podocarpusflavone A. Compounds bearing two methoxyl substituents include ginkgetin, isoginkgetin, and 5′-methoxybilobetin, whereas sciadopitysin contains three such groups. In addition to methylation, biflavonoids in ginkgo, much like monomeric flavonoids, can undergo glycosylation. So far, several glycosylated forms have been reported, including 7″-*O*-β-D-glucosyl-ginkgetin, 7′-*O*-β-D-glucosyl-isoginkgetin, and amentoflavone 7″-*O*-β-D-glucopyranoside (Table 5) (Šamec et al., 2022).

Despite the long-standing recognition of ginkgo as a natural source of 3′,8″-biflavonoids, published reports on their occurrence have been sporadic and largely restricted to leaf tissues (Šalić et al., 2024; Su et al., 2022; Yang, 2018). Only a limited number of studies have reported the presence of 3′,8″-biflavones in other organs, including stems (Pandey et al., 2014), male flowers (Li et al., 2019) and fruits (Shen et al., 2022; Chen et al., 2021; Pandey et

al., 2014; Zhou et al., 2012). Consequently, a comprehensive analysis of the major 3′,8″-biflavones across different ginkgo organs has been lacking. This gap can be attributed, in part, to the previous unavailability of appropriate analytical standards and the absence of an optimized method capable of reliably separating, detecting, and quantifying these structurally similar dimers. As a consequence, most earlier studies reported the presence of only a limited number of biflavonoids, typically two (Pandey et al., 2014) or three compounds (Kaur et al., 2011), and only rarely extended the analysis to include four compounds (Lei et al., 2021; Wang et al., 2019; Beck & Stengel, 2016).

To address this limitation, the present doctoral research (Article 2) provides the first systematic overview of the distribution of key 3′,8″-biflavonoids across a wide range of ginkgo tissues, including leaf blades, petioles, seed components (exotesta, endotesta, and seed petioles), tree bark, twig bark, debarked twigs, and buds. This was achieved using a specifically optimized HPLC-DAD method for the chromatographic separation, identification, and quantification of the five major 3′,8″-biflavones. Furthermore, as ginkgo leaves represent the richest source of 3′,8″-biflavonoids, a systematic evaluation of different drying techniques was conducted to assess their impact on the preservation and overall yield. Three commonly applied drying methods - air-drying, oven-drying, and freeze-drying were compared in order to identify the most suitable approach for the sample preparation in subsequent analyses (Article 4).

1.4. 3′,8″-biflavones: biological activity, accumulation dynamics and ecological drivers

The content of specialized metabolites may vary considerably depending on factors such as plant age, growth location, sex, and environmental conditions (Pant et al., 2021); however, data on the synthesis and accumulation dynamics of 3′,8″-biflavones remain limited (Šamec et al., 2022). To date, most studies investigating metabolite fluctuations in response to abiotic and biotic factors have focused predominantly on monomeric flavonoids in ginkgo leaves (Guo et al., 2023; Zhao et al., 2020; Rimkiene et al., 2017; Yao et al., 2012), leaving the regulation and accumulation dynamics of biflavonoids largely unexplored.

Earlier studies consistently indicate that the highest concentrations of 3′,8″-biflavonoids occur in ginkgo leaves (Lei et al., 2021; Beck & Stengel, 2016; Wang et al., 2020; Kaur et al., 2011; Lin et al., 2008; Wollenweber et al., 1998), followed by other external organs such as stems (Pandey et al., 2014), flowers (Li et al., 2019), and fruits (Shen et al., 2022; Chen et al., 2021; Pandey et al., 2014; Zhou et al., 2012). Nevertheless, existing research remains fragmented and rarely encompasses the full spectrum of biflavonoids present in ginkgo. Current

findings suggest that bilobetin, ginkgetin, isoginkgetin, and sciadopitysin are the predominant dimers in leaves, with sciadopitysin typically occurring in the highest amounts (Lei et al., 2021).

MALDI mass spectrometric imaging has further revealed that biflavonoids are primarily localized on the leaf surface, particularly within the lower epidermis, supporting their putative role in surface-associated defense mechanisms (Beck & Stengel, 2016). In addition to leaves, biflavonoids have been detected in stems, flowers, and fruits, with substantial variation observed between different organs and even between male and female trees (summarized by Šamec et al. (2022)). Notably, sciadopitysin levels may exceed those of amentoflavone by up to 200-fold, and older trees tend to accumulate higher overall levels of biflavonoids than younger individuals (Pandey et al., 2014). Among the 3′,8″-linked biflavonoids, sciadopitysin consistently emerges as the most abundant compound in ginkgo leaves (Šalić et al., 2024; Lei et al., 2021; Wang et al., 2019). Furthermore, biflavonoids have been identified in the sarcotesta (fruit exocarp) (Zhou et al., 2012), prompting the development of efficient industrial extraction strategies to recover high-purity biflavonoids from this otherwise discarded biomass (Shen et al., 2022).

Despite the fact that most 3′,8″-biflavones were isolated and structurally characterized several decades ago, their early identification was often constrained by limited analytical resolution, the absence of authentic reference standards, and inconsistent nomenclature. As a consequence, some structural assignments remain uncertain, with identical names occasionally applied to distinct isomers and vice versa (Šamec et al., 2024).

Scientific interest in biflavonoids has been steadily increasing due to their promising potential in the development of novel therapeutics for various diseases (Liu et al., 2021). The accumulation of 3′,8″-biflavones in plants is likely linked to defense against biotic stress, as several studies have demonstrated their strong antimicrobial properties. Bilobetin and 4-*O*-methylamentoflavone were identified for the first time in *Taxus baccata* needles, and biflavones from *T. baccata* and *G. biloba* showed notable antifungal activity against *Alternaria alternata*, *Fusarium culmorum*, and *Cladosporium oxysporum*, with bilobetin exhibiting particularly strong growth inhibition and complete suppression of germ tube development at higher concentrations, while ginkgetin and 7-*O*-methylamentoflavone were especially effective against *A. alternata* and induced detectable cell wall alterations (Krauze-Baranowska and Wiwart, 2003). Furthermore, biflavonoids were shown to suppress aflatoxin B1 and B2 production by *Aspergillus flavus* and *A. parasiticus*, exhibiting greater activity at lower concentrations than monomeric flavonoids, suggesting that the dimeric structure enhances

bioactivity. The authors proposed that biflavonoids could serve as potential agents for aflatoxin control (Gonçalez et al., 2001).

Beyond their antimicrobial functions, biflavonoids have been shown to influence photosynthetic processes, including ATP synthesis and electron transport in chloroplasts, by targeting key components of the photosynthetic electron transport chain (Aguilar et al., 2008). Céspedes et al. (2001) demonstrated a concentration-dependent inhibition of photophosphorylation by several biflavonoids, highlighting their ability to modulate energy metabolism in plants. In addition, amentoflavone isolated from *Selaginella tamariscina* was identified as a highly selective and potent natural agent that effectively eliminates *Microcystis aeruginosa*, where it disrupts cell membrane integrity, underscoring its potential as an environmentally friendly strategy for controlling harmful cyanobacterial blooms (Lee et al., 2020). Furthermore, amentoflavone isolated from *Byrsonima crassa* exhibited dose-dependent and selective effects on tomato seedling development, stimulating shoot elongation at low concentrations and suggesting a role as a natural regulator of plant growth (De Almeida et al., 2007).

Despite the growing body of evidence supporting the ecological and pharmacological relevance of biflavonoids, their precise biological functions and physiological roles in plants - particularly those of 3′,8″-biflavones - remain insufficiently understood. Studies addressing their accumulation patterns and regulatory dynamics are scarce and limited in scope, with most prior research focusing on monomeric flavonoids. Consequently, the seasonal dynamics of biflavonoid biosynthesis and accumulation remain largely unexplored. As seasonal fluctuations in secondary metabolite levels can provide valuable insights into their physiological and ecological roles, this doctoral dissertation presents the first continuous, season-long assessment of the accumulation dynamics of the major 3′,8″-biflavones in ginkgo leaves, spanning from the onset of spring growth to late autumn (Article 3).

To further extend this seasonal perspective, the content of 3′,8″-biflavones was additionally examined during autumnal leaf senescence and in naturally fallen leaves. As both leaf types represent plant-derived biowaste, this comparison aimed to evaluate their potential as sustainable and eco-friendly raw materials for the recovery of bioactive biflavonoids (Article 6). Finally, the phytochemical composition and biological activity of ginkgo infusions prepared from spring leaves, yellow autumn leaves, and fallen autumn leaves were comparatively assessed to explore their functional potential (Article 5).

1.5. Objectives and hypotheses

The objectives of this dissertation are to measure the *in vitro* activity of 3′,8″-biflavones from ginkgo in comparison to their monomeric subunits; to optimize a method for their extraction, separation, and quantification; to determine their content in various ginkgo tissues; and to assess the dynamic changes in 3′,8″-biflavones in ginkgo leaves throughout the entire vegetation period.

To achieve these objectives, the following hypotheses were formulated:

1. The dimerization of flavonoids affects their biological activity.
2. The accumulation of biflavonoids in ginkgo is tissue-specific.
3. The accumulation of biflavonoids changes across growth stages.

To address these objectives and test the proposed hypotheses, the following research articles were produced within the scope of this dissertation.

Main articles:

1. **Jurčević Šangut, I**., Šarkanj, B., Karalija, E., & Šamec, D. (2023). A Comparative Analysis of radical Scavenging, Antifungal and Enzyme Inhibition Activity of 3′,8″-Biflavones and Their Monomeric Subunits. *Antioxidants*, *12*, 1854. https://doi.org/10.3390/antiox12101854
2. Kovač Tomas, M., **Jurčević, I.**, & Šamec, D. (2023). Tissue-Specific Profiling of Biflavonoids in Ginkgo (*Ginkgo biloba* L.). *Plants*, *12*, 147. https://doi.org/10.3390/plants12010147
3. **Jurčević Šangut, I**., & Šamec, D. (2024). Seasonal Variation of Polyphenols and Pigments in Ginkgo (*Ginkgo biloba* L.) Leaves: Focus on 3′,8″-Biflavones. *Plants*, *13*, 3044. https://doi.org/10.3390/plants13213044

Additional articles:

4. **Jurčević Šangut, I**., Pavličević, L., & Šamec, D. (2024) Influence of Air Drying, Freeze Drying and Oven Drying on the Biflavone Content in Yellow Ginkgo (*Ginkgo biloba* L.) Leaves. *Applied Sciences*, *14*, 2330. https://doi.org/10.3390/app14062330
5. **Jurčević Šangut, I**., Šola, I., & Šamec, D. (2024) Neuroprotective, Anti-Hyperpigmentation, and Anti-Diabetic Effects in Ginkgo Leaf Infusion from Green and Yellow Leaves. *Applied Sciences*, 14, 10231. https://doi.org/10.3390/app142210231

6. **Jurčević Šangut, I.**, & Šamec, D. (2025) From Waste to Resource: Valorization of Yellow Ginkgo Leaves as a Source of Pharmacologically Relevant Biflavonoids. *Applied Sciences*, *15*, 11436. https://doi.org/10.3390/app152111436

To test how dimerization affects the biological activity of flavonoids (H1, O1), a series of *in vitro* analyses was conducted using major 3′,8″-biflavone standards (amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin) and their corresponding monomeric subunits (apigenin, genkwanin, and acacetin). The compounds were evaluated for their antioxidant activity based on DPPH radical scavenging capacity, as well as for their antifungal activity against selected mycotoxigenic fungi of importance for food safety (*Alternaria alternata*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Fusarium graminearum*, and *Fusarium verticillioides*). Furthermore, their inhibitory effects were evaluated against a set of enzymes of key relevance to human health, including acetylcholinesterase, tyrosinase, α-amylase, and α-glucosidase. The results of these investigations are summarized in Article 1. Article 2 provides the first insight into the tissue-specific distribution of major 3′,8″-biflavones (amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin) in ginkgo. In addition, this study presents a newly developed and optimized HPLC-DAD method for the simultaneous separation and quantification of these five major biflavones, thereby addressing hypothesis H2 and objective O3. In order to optimize the preservation of 3′,8″-biflavones, Article 4 identifies the most suitable drying method for ginkgo leaves, contributing to objective O2. Moreover, Article 5 examines the *in vitro* biological activities of infusions prepared from green and yellow ginkgo leaves, while Article 6 explores the potential use of yellow ginkgo leaves as an economical and abundant source of 3′,8″-biflavones. Finally, Article 3 investigates the seasonal dynamics of biflavone accumulation in ginkgo leaves throughout the entire vegetation period (H3, O4). This study was designed to evaluate changes in biflavone levels during plant development and to assess their accumulation patterns over the course of the vegetation period.

2. Discussion

2.1. Dimeric architecture as the key to the biological efficacy of biflavonoids

The chemical structure of flavonoids is a central determinant of their biological activity. Among the structural features that critically modulate their physicochemical properties and bioactivity, dimerization represents a particularly distinctive and functionally relevant modification (Zou et al., 2025; Duman et al., 2025; Lopes et al., 2024; Menezes & Campos, 2021; X. Li et al., 2019). Accordingly, the first hypothesis of this dissertation proposed that 3′,8″-dimerization fundamentally affects flavonoid biological activity. This hypothesis was rigorously tested in Article 1 through a systematic comparison of antioxidant, antifungal, and enzyme inhibitory activities of structurally related flavonoid pairs, including authentic standards of 3′,8″-biflavonoids and their corresponding monomeric counterparts. The results unequivocally confirmed the hypothesis, demonstrating that dimerization does not merely intensify biological activity but rather selectively reshapes bioactivity, leading to enhancement, attenuation, or complete loss of specific bioactivities depending on the biological target.

A substantial body of research has documented the diverse biological activities of biflavonoids, particularly in the context of their potentially beneficial effects on human health, as summarized in Table 3. To date, the majority of reported activities have been established predominantly through *in vitro* assays, which continue to represent the dominant experimental approach for evaluating biflavonoid bioactivity. However, an increasing number of recent studies have expanded this scope by incorporating *in vivo* models and *in silico* analyses, providing deeper insights into bioavailability, molecular targets, and structure-activity relationships.

In terms of antioxidant capacity, neither the monomeric flavones nor their corresponding biflavonoid dimers exhibited substantial radical-scavenging activity in the DPPH assay. Notably, both the biflavonoids and monomeric subunits consistently showed weak or negligible antioxidant effect even at high tested concentrations (1 mg/mL), indicating a limited capacity for direct free-radical scavenging under the applied experimental conditions. These observations are in good agreement with previous reports, which likewise identified 3′,8″-biflavones as the flavonoid subclass exhibiting the weakest antioxidant activity among structurally related flavonoids (Kang et al., 2005; Bedir et al., 2002). Moreover, the correlation matrix presented in Article 3 revealed a strong negative correlation between the content of 3′,8″-biflavones in ginkgo extracts and DPPH radical-scavenging activity. This relationship suggests

that higher biflavonoid levels are associated with lower DPPH scavenging capacity, supporting the conclusion that 3′,8″-biflavones possess limited direct antioxidant activity, at least in cell-free assay systems.

In contrast, the study by Li et al. (2019) demonstrated that 3′,8″-dimerization enhances the antioxidant activity of flavonoids, as isoginkgetin exhibited a higher electron-transfer capacity and stronger antioxidant activity than its monomeric precursor, acacetin. Furthermore, amentoflavone isolated from *Nandina domestica* has been reported to exhibit pronounced antioxidant activity in both DPPH and ABTS *in vitro* assays, acting in a clear concentration-dependent manner (Bajpai et al., 2019). However, although certain plant-derived biflavonoids have demonstrated notable i*n vitro* antioxidant activity (Andrade et al., 2018), their efficacy is generally lower than that of corresponding monomeric flavonoids, indicating that dimerization does not necessarily enhance radical-scavenging capacity (Xiao et al., 2019). Additionally, ABTS, FRAP, and CUPRAC analyses of different biflavonoid dimers showed that compounds with a higher number of hydroxyl (-OH) groups exhibited greater antioxidant activity. These findings may suggest that substitution patterns, particularly the number and position of -OH groups, may play a more decisive role in antioxidant potential than dimerization itself, which appears to modulate activity in a target- and mechanism-dependent manner (Sabudak et al., 2019).

According to the review by Zou et al. (2025), the enhanced antioxidant activity of flavonoid dimers can be explained by several interconnected structural and electronic mechanisms. First, dimerization may increase the electron-transfer potential of the molecule compared with its monomeric counterparts, thereby facilitating more efficient neutralization of free radicals (Li et al., 2019). In addition, the conjugated systems formed by linking two flavonoid units enable a more favorable distribution of electron density, contributing to the stabilization of phenoxyl radicals and more effective interactions with reactive oxygen species (Deng et al., 2023). Furthermore, the higher number of phenolic hydroxyl groups in flavonoid dimers increases their ability to form multiple hydrogen bonds, further stabilizing radical–molecule interactions and potentially enhancing antioxidant efficacy (Cai et al., 2019). The authors also emphasize that the spatial organization and reduced rotational freedom of dimeric structures can influence the reaction kinetics, thereby further modulating their antioxidant activity.

A broad spectrum of antimicrobial activities contributes to the overall biological activity of biflavonoids, with dimerization playing a key role in modulating and often enhancing these effects as summarized by Lopes et al. (2024). In our work, we demonstrated the impact of 3′,8″-biflavones on several mycotoxigenic fungal species relevant to food safety and human health.

The results of the antifungal assays showed that antifungal efficacy is highly dependent on both the fungal species and the applied concentration, and that 3′,8″-dimerization represents an important structural factor shaping both activity intensity and selectivity.

A particularly strong growth inhibition of *Fusarium graminearum* was observed already at very low concentrations, with biflavonoids exhibiting markedly higher activity than the corresponding monomeric flavonoids. This pattern suggests that dimerization may enhance antifungal efficacy against certain *Fusarium* species, which is consistent with previous reports highlighting the pronounced sensitivity of these pathogens to biflavonoids (Krauze-Baranowska & Wiwart, 2003). In contrast, for *Fusarium verticillioides* and species of the genus *Aspergillus*, antifungal effects were weaker and generally apparent only at higher concentrations, displaying distinct concentration-dependent trends that further emphasize the selective nature of biflavonoid activity. No significant inhibition of *Alternaria alternata* was observed within the tested concentration range, in agreement with literature reports indicating that substantially higher concentrations are required to achieve antifungal effects against this species (Krauze-Baranowska & Wiwart, 2003).

Dimeric flavonoids are thought to exert their antifungal effects through multiple complementary mechanisms, including the formation of complexes with soluble proteins in the fungal cell wall, as well as through their pronounced lipophilicity, which enables interaction with and disruption of fungal membrane integrity (Menezes & Campos, 2021). Biflavonoids inhibit fungal growth and spore germination and, in *Candida* species, have also been reported to affect biofilm formation (Lee et al., 2024). Overall, these findings confirm that biflavonoids do not act as broad-spectrum antifungal agents, but rather exhibit species- and concentration-dependent activity, in which the dimeric flavonoid architecture can, in specific cases, significantly contribute to antifungal efficacy.

In the third part of Article 1, we examined the effects of biflavonoids and their corresponding monomeric flavonoids on the activity of enzymes of major relevance to human health. These included acetylcholinesterase, whose inhibition leads to increased acetylcholine levels and is associated with improved cognitive function in Alzheimer's disease (Moss, 2020); α-amylase and α-glucosidase, key enzymes involved in carbohydrate metabolism and the regulation of postprandial hyperglycemia (Jha et al., 2025); and tyrosinase, an enzyme whose overactivity or overexpression is linked to hyperpigmentation disorders and has also been associated with melanoma development (Baber et al., 2023).

In our study, enzyme inhibition was shown to be strongly structure-dependent, with 3′,8″-dimerization selectively modulating enzymatic activity. Among the tested compounds,

the biflavonoids ginkgetin and isoginkgetin exhibited the strongest inhibition of acetylcholinesterase, which is consistent with the established use of ginkgo in the treatment of cognitive disorders (Muratori et al., 2025; Noor-E-Tabassum et al., 2022) and supports the neuroprotective potential of these dimers. Similarly, Sadeghi et al. (2024) identified ginkgetin as a potent acetylcholinesterase inhibitor using molecular docking, with its inhibitory potential confirmed by parallel *in vitro* assays, highlighting ginkgetin as a promising natural candidate for modulation of the cholinergic system in the context of Alzheimer's disease.

In contrast, monomeric flavonoids were more effective tyrosinase inhibitors than biflavones, although amentoflavone displayed moderate activity, indicating that dimerization does not uniformly enhance inhibition across different enzymatic targets. The study by Nasr Bouzaiene et al. (2016) demonstrated that genkwanin exhibits pronounced antiproliferative effects in B16F10 melanoma cells by disrupting cell cycle progression and inducing apoptosis, while simultaneously reducing melanin synthesis through inhibition of tyrosinase activity. These findings are consistent with our results, in which genkwanin showed significant tyrosinase inhibitory activity among other monomers, further supporting its potential role in the regulation of melanogenesis and in hyperpigmentation-related processes.

In the context of carbohydrate metabolism, amentoflavone emerged as the most potent inhibitor of both α-amylase and α-glucosidase, in agreement with previous *in vitro* (Li et al., 2023) and molecular docking studies that identify it as a strong noncompetitive inhibitor with potential for regulating postprandial hyperglycemia (Swargiary et al., 2023). Overall, these findings demonstrate that biflavonoids and monomeric flavonoids from ginkgo exhibit distinct, enzyme-specific inhibitory profiles, underscoring the critical role of structural features in determining their biological relevance.

Overall, the results demonstrate that 3′,8″-dimerization represents a key structural factor that selectively modulates flavonoid bioactivity rather than uniformly enhancing it. Dimerization reshapes biological function in a target-dependent manner, strengthening antifungal and selected enzyme-inhibitory effects while attenuating direct antioxidant and tyrosinase-inhibitory activities. These findings confirm that biflavonoid efficacy is governed by a fine balance between dimeric architecture, substitution pattern, and biological context.

2.2. Tissue-specific differences as a determinant of the biflavonoid metabolic profile of ginkgo

The second hypothesis of this doctoral dissertation proposed that biflavonoid accumulation in ginkgo is tissue-specific, reflecting organ specialization and plant-environment interactions. Firstly, as we reported in Article 2, we developed and validated an HPLC–DAD method that enables the simultaneous identification and quantification of the five major biflavonoids present in ginkgo - amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin. The developed method provides good chromatographic resolution, allowing reliable separation of the structural isomers ginkgetin and isoginkgetin, which in earlier studies were sometimes reported as a single chromatographic peak (Beck & Stengel, 2016; Kaur et al., 2011).

The results presented in Article 2 unequivocally confirm the second hypothesis, revealing pronounced differences in both the abundance and composition of major 3′,8″-biflavones across distinct ginkgo tissues. Using a newly optimized HPLC-DAD method, we demonstrated that biflavonoids accumulate exclusively in tissues in direct contact with the external environment. Furthermore, this study provides the first comprehensive overview of the distribution of 3′,8″-biflavones across such a broad range of ginkgo tissues, encompassing ten distinct plant parts. Leaf blades showed by far the highest biflavonoid content, followed by leaf petioles, twig bark, buds, and the sarcotesta, whereas no biflavonoids were detected in seeds, nutshells, or internal twig tissues. This pronounced spatial pattern is consistent with MALDI-MS imaging studies, which have demonstrated that biflavonoids predominantly accumulate in epidermal and subepidermal layers of ginkgo leaves (Beck & Stengel, 2016). Similarly, Li et al. (2018) reported the successful *in situ* detection and localization of biflavonoids in ginkgo leaves using MALDI-FT-ICR and LDI-MS, revealing their preferential accumulation in tissues directly exposed to the external environment. Comparable localization has also been observed in the outer tissues of *Psilotum nudum* rhizomes, further supporting the notion that biflavonoids may have a role in plant defense (Šamec et al., 2019).

The distribution of individual biflavonoids further reflects tissue specialization. In leaves, the most abundant compound was sciadopitysin - consistent with previous reports (Wang et al., 2019; Pandey et al., 2014), followed by isoginkgetin and ginkgetin, while amentoflavone was present in the lowest amounts. In leaf blades, amentoflavone occurred at markedly lower concentrations than the other major 3′,8″-biflavones, with levels approximately 15-27-fold lower than those of sciadopitysin, isoginkgetin, and ginkgetin. Although amentoflavone elutes

first during HPLC analysis and has frequently been reported in earlier studies, its low abundance in leaf blades, often close to the detection limit, may have contributed to the historical underrepresentation of biflavonoids in ginkgo extracts, particularly in studies lacking adequate reference standards and optimized separation methods. This distribution may suggest enhanced *O*-methylation activity in photosynthetic tissues, as sciadopitysin and ginkgetin represent more highly methylated derivatives of amentoflavone. In contrast, in twig bark, tree bark, and buds, amentoflavone predominated, whereas highly methylated dimers occurred only in minor amounts or were below the detection limit. Notably, the occurrence of amentoflavone in ginkgo stems reported by Pandey et al. (2014) is in line with its preferential accumulation in supporting and protective tissues, a distribution pattern also observed in other plant species producing biflavonoids (Doan et al., 2022; Ndongo et al., 2015).

The presence of biflavonoids in the seed sarcotesta and petioles emphasizes their preferential accumulation in outer and exposed plant tissues. In this tissue, isoginkgetin was the most abundant biflavonoid, accompanied by detectable levels of the other major 3′,8″-biflavones. Industrial-scale isolation of biflavonoids from the sarcotesta confirms both their high local concentrations and their relevance as a potential source of these compounds (Shen et al., 2022). Beyond vegetative organs, biflavonoids have also been reported in reproductive tissues of ginkgo. Li et al. (2019) identified amentoflavone, sciadopitysin, bilobetin, and isoginkgetin in male flowers of ginkgo, broadening their known tissue distribution.

Taken together, these results demonstrate that 3′,8″-biflavonoids in ginkgo exhibit a highly structured, tissue-specific accumulation pattern, with a clear preference for external and environmentally exposed organs. While this spatial organization strongly suggests a role in protective and adaptive plant functions, the present findings primarily establish a robust biochemical and anatomical framework within which the physiological significance of these compounds can now be systematically explored.

2.3. Seasonal dynamics as a regulator of biflavonoid accumulation

The third hypothesis proposed that biflavonoid accumulation changes throughout the growing season, potentially reflecting developmental transitions and environmental cues. The results presented in Article 3 confirm this hypothesis and provide new insights into the seasonal behavior of 3′,8″-biflavonoids in ginkgo. Throughout the entire vegetation period, the relative proportions of the five major 3′,8″-biflavones remained remarkably stable, following the consistent trend amentoflavone < bilobetin < ginkgetin < isoginkgetin < sciadopitysin. This compositional stability may indicate a tightly regulated biosynthetic relationship between

monomeric precursors and subsequent methylation steps. In contrast, the total biflavonoid content increased steadily from early spring to late autumn, reaching its maximum immediately prior to leaf senescence.

This seasonal accumulation pattern aligns with previous reports describing increased flavonoid levels in the later stages of the growing season (Zou et al., 2019; Rimkiene et al., 2017; Xu et al., 2014), although these studies primarily focused on monomeric flavonoid aglycones. Across all developmental stages, sciadopitysin remained the dominant biflavonoid, reflecting sustained and robust methylation activity in mature leaves. Similarly, Wu et al. (2021), who quantified eleven flavonoids in ginkgo leaves collected from nineteen regions across China, reported sciadopitysin as the most abundant compound in all samples, followed by isoginkgetin, ginkgetin, bilobetin, and amentoflavone, indicating a conserved biflavonoid composition despite pronounced geographic variability.

The strong negative correlation observed between chlorophylls and biflavonoids suggests that biflavonoids may be associated with physiological processes accompanying leaf maturation and senescence. Their progressive accumulation coincides with increasing exposure to abiotic stresses, such as enhanced UV-B radiation and oxidative pressure toward the end of the growing season (Guo et al., 2023; Mao et al., 2023; Zhao et al., 2020). Given their high relative abundance in mature and senescent leaves, and their predominance over carotenoids at late-season stages, 3′,8″-biflavones may substantially contribute to the characteristic yellow coloration of ginkgo foliage, consistent with early reports identifying ginkgetin as a major yellow pigment (Sugasawa, 1964).

Previous investigations of seasonal biflavonoid variation in other species, such as *Taxus wallichiana,* reported irregular or inconsistent patterns, likely due to methodological limitations or insufficient temporal resolution (Wu et al., 2021). In contrast, the present study provides the first high-resolution, season-long reconstruction of biflavonoid dynamics in ginkgo, demonstrating a clear and gradual accumulation trend supported by robust analytical methodology. These data confirm that the vegetative stage is a major determinant of biflavonoid levels, reflecting an interplay between developmental programming and environmental stress adaptation.

In addition to seasonal regulation, post-harvest processing represents an important factor influencing the practical utilization of ginkgo biomass. Besides the extraction procedure itself, the drying method of ginkgo plant material is critical for preserving an optimal phytochemical composition. In Article 3, three commonly applied drying approaches - air-drying, oven-drying, and freeze-drying, were systematically compared. The results demonstrated that none of these

drying methods caused significant changes in the contents of total polyphenols, total flavonoids, or 3′,8″-biflavones. This stability indicates that biflavonoid profiles established during leaf development and senescence remain largely preserved during drying. Importantly, these findings support the use of simpler and more cost-effective drying techniques, such as air drying, without compromising phytochemical quality, which is particularly relevant for large-scale and industrial processing of ginkgo biomass.

According to these findings, yellow autumn ginkgo leaves represent a particularly rich natural reservoir of 3′,8″-biflavones, as their concentrations markedly increase toward the end of the vegetation period. Article 6 further examined the biflavonoid profiles of both yellow leaves still attached to the tree and those naturally fallen to the ground, demonstrating that naturally shed leaves retain, and in some cases even exceed, the biflavonoid levels observed in on-tree yellow leaves. These results indicate that autumn ginkgo foliage, traditionally regarded as biological waste, may represent a readily accessible and sustainable source of pharmaceutically relevant biflavonoids. At the same time, they underscore the importance of evaluating compositional variability and safety aspects when considering senescent leaves as raw material for further applications.

Ginkgo infusions, more widely known as ginkgo tea, are traditionally prepared from green ginkgo leaves. Building on this practice, the biological activity of infusions prepared from spring and autumn ginkgo leaves was evaluated in Article 5 to assess whether seasonal leaf senescence affects phytochemical composition and bioactivity. In addition to spring green leaves, infusions obtained from yellow autumn leaves were included for direct comparison. In contrast to organic solvent extracts, biflavonoids were not detected in any of the aqueous infusions, consistent with their low water solubility and the well-established requirement for organic solvents, such as higher concentrations of methanol or ethanol, for efficient extraction of 3′,8″-biflavones (Medvedec and Šamec, 2026). Similarly, Su et al. (2022) reported the presence of biflavonoids exclusively in ginkgo wine, but not in aqueous infusions, further confirming the strong solvent dependence of biflavonoid extractability.

Despite the absence of biflavonoids, infusions prepared from yellow autumn leaves contained a 3.2-fold higher total polyphenol content and approximately threefold higher total flavonoid levels compared to spring leaf infusions. As a direct consequence of this enriched phenolic profile, autumn leaf infusions exhibited markedly enhanced biological activity, including a 4.8-fold higher antioxidant capacity and significantly stronger enzyme inhibition. In particular, tyrosinase inhibition exceeded 70%, substantially surpassing the activity observed for spring leaf infusions. These findings are consistent with the study by Su et al. (2022b), who

likewise reported significantly higher flavonoid contents and antioxidant activity in teas prepared from naturally senescent yellow ginkgo leaves. Furthermore, Klomsakul et al. (2022) demonstrated concentration-dependent tyrosinase inhibition by ginkgo leaf tea extracts, linking their phenolic composition to enzymatic activities relevant to pigmentation and oxidative stress. In the context of skin health, earlier studies have shown that flavonoids from ginkgo infusions exert antioxidant and photoprotective effects, contributing to protection against UV-induced skin damage and preservation of skin barrier function (dal Belo et al., 2011; dal Belo et al., 2009).

Collectively, these findings indicate that, despite the absence of biflavonoids, elevated levels of monomeric flavonoids and other phenolic constituents are the primary contributors to the enhanced bioactivity of autumn leaf infusions. Yellow autumn ginkgo leaves thus emerge as an underutilized and potentially sustainable raw material with considerable promise for functional, nutraceutical, and therapeutic applications. However, their practical use must be carefully evaluated in light of the concomitant increase in certain toxic constituents, such as ginkgolic acids, during leaf senescence, which necessitates appropriate processing, standardization, and toxicological assessment prior to broader application.

Finally, according to Kulić et al. (2022), the production of the standardized ginkgo extract EGb 761® is based on a patented multi-step process designed to ensure consistent quality. During this process, biflavonoids are intentionally removed at an early stage, where they co-precipitate with chlorophylls and most ginkgolic acids and are discarded as waste. Consequently, although biflavonoids naturally occur in ginkgo leaves, they are largely absent from EGb 761®, indicating that their biological and pharmacological roles are not represented in the activity profile of this standardized extract. Consistent with this, analyses of commercial dietary supplements confirm a predominance of monomeric flavonoids, primarily quercetin and kaempferol, as shown by CE–MS analysis (Johnson & Lunte, 2016).

3. Conclusions

1. This dissertation demonstrates that 3′,8″-dimerization represents a critical structural determinant of flavonoid bioactivity. Comparative *in vitro* analyses clearly show that dimerization does not uniformly enhance biological effects but selectively modulates them in a target-dependent manner. Certain 3′,8″-biflavones exhibited stronger inhibition of specific enzymes and enhanced antifungal activity, whereas in other cases monomeric flavonoids were more effective. Importantly, all investigated 3′,8″-biflavones displayed weak radical-scavenging activity, indicating that their biological relevance may not primarily be linked to direct antioxidant mechanisms but rather to alternative modes of action.
2. A reliable HPLC-DAD analytical method was successfully developed and optimized, enabling the separation, detection, and accurate quantification of all five major 3′,8″-biflavones. This methodological advance provided the analytical foundation for detailed tissue-specific and seasonal profiling of biflavonoids in ginkgo.
3. Biflavonoid accumulation in ginkgo is highly tissue-specific, confirming the second hypothesis of this dissertation. 3′,8″-biflavones were detected exclusively in tissues directly exposed to environmental factors, with the highest concentrations in leaf blades, followed by petioles, bark, buds, and sarcotesta. This distribution strongly supports a functional role in environmental interaction and stress protection.
4. The relative proportions of individual 3′,8″-biflavones remained remarkably stable across tissues and throughout the growing season, following the conserved order amentoflavone<bilobetin<ginkgetin<isoginkgetin<sciadopitysin. This compositional stability suggests tightly regulated biosynthetic and methylation pathways underlying biflavonoid formation in ginkgo.
5. Seasonal analysis revealed a pronounced increase in total biflavonoid content from spring to late autumn, with maximum concentrations observed in yellow senescent leaves. This pattern confirms the third hypothesis and indicates that biflavonoid accumulation is developmentally regulated and closely linked to leaf maturation and senescence.
6. Post-harvest processing was shown to have minimal impact on biflavonoid integrity, as air-drying, oven-drying, and freeze-drying preserved total polyphenols, total flavonoids, and 3′,8″-biflavone contents equally well. These results support the use of simple, cost-

effective drying strategies, particularly relevant for large-scale and industrial processing of ginkgo biomass.

7. Yellow autumn leaves, including naturally fallen leaves, were identified as exceptionally rich sources of 3′,8″-biflavones, often matching or exceeding levels found in leaves still attached to the tree. These findings highlight ginkgo leaf litter as a sustainable, non-invasive, and economically attractive source of pharmaceutically relevant biflavonoids.
8. Aqueous infusions prepared from ginkgo leaves did not contain biflavonoids, reflecting their low water solubility. Nevertheless, infusions from autumn leaves exhibited significantly higher polyphenol and flavonoid contents, stronger antioxidant capacity, and enhanced enzyme inhibition compared to spring leaf infusions. These effects were driven by monomeric flavonoids and other phenolic constituents, underscoring the importance of seasonal leaf chemistry for traditional ginkgo preparations.

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5. Appended publications

5.1. Main articles

5.1.1. A Comparative Analysis of Radical Scavenging, Antifungal and Enzyme Inhibition Activity of 3'-8''-Biflavones and Their Monomeric Subunits

antioxidants

MDPI

Article

A Comparative Analysis of Radical Scavenging, Antifungal and Enzyme Inhibition Activity of 3′-8″-Biflavones and Their Monomeric Subunits

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Abstract: Biflavonoids are dimeric forms of flavonoids that have recently gained importance as an effective new scaffold for drug discovery. In particular, 3′-8″-biflavones exhibit antiviral and antimicrobial activity and are promising molecules for the treatment of neurodegenerative and metabolic diseases as well as cancer therapies. In the present study, we directly compared 3′-8″-biflavones (amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin) and their monomeric subunits (apigenin, genkwanin, and acacetin) and evaluated their radical scavenging activity (with DPPH), antifungal activity against mycotoxigenic fungi (*Alternaria alternata*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Fusarium graminearum*, and *Fusarium verticillioides*), and inhibitory activity on enzymes (acetylcholinesterase, tyrosinase, α-amylase, and α-glucosidase). All the tested compounds showed weak radical scavenging activity, while antifungal activity strongly depended on the tested concentration and fungal species. Biflavonoids, especially ginkgetin and isoginkgetin, proved to be potent acetylcholinesterase inhibitors, whereas monomeric flavonoids showed higher tyrosinase inhibitory activity than the tested 3′-8″-biflavones. Amentoflavone proved to be a potent α-amylase and α-glucosidase inhibitor, and in general, 3′-8″-biflavones showed a stronger inhibitory potential on these enzymes than their monomeric subunits. Thus, we can conclude that 3′-8″-dimerization enhanced acetylcholinesterase, α-amylase, and α-glucosidase activities, but the activity also depends on the number of hydroxyl and methoxy groups in the structure of the compound.

Keywords: 3′-8″-biflavones; amentoflavone; bilobetin; ginkgetin; isoginkgetin; sciadopitysin; apigenin; genkwanin; acacetin

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1. Introduction

Flavonoids have been the focus of scientific attention for more than 40 years and 8000 different structures have been described to date [1]. However, they are a large group of specialized metabolites whose biological function in plants and biological activity are strongly influenced by molecular structure [2]. The basic structure of flavonoids is presented in Figure 1a. In general, flavonoids occur as aglycons or in conjugated form. Aglycons are often hydroxylated at the C3, C5, C7, C3′, C4′, and C5′ positions, while some of these hydroxyl groups may also be methylated, acetylated, or sulfated [3]. Prenylation occurs directly on a carbon atom in the aromatic rings, but *O*-prenylation has also been found [3]. The hydroxylation of C5, C7, C3′, and C4′ and geranylation or prenylation at C6 have been extensively studied to increase the bacterial inhibition of flavonoids, while methoxylation at C3′ and C5 decreases the antibacterial activity of flavonoids [4]. According to a study by Zhang et al. [5], the 2,3-double bond, 4-keto groups, 3′,4′-catechol structure, and 3-hydroxyl in the flavonoid scaffold play an important role in the antioxidant behavior, while the cell proliferation assay showed low cytotoxicity for 3-*O*-methylquercetin. According to

Boozari et al. [6], the prenyl group in the C8 position plays an important role in biological effects such as antimicrobial, cytotoxic, enzyme inhibitory, and estrogenic activity. The resveratrol residue (A or B ring) in combination with lavandulyl flavanones in the structure of flavonoids may enhance their cytotoxic effect, while the presence of prenyl groups in any position of the flavonoid backbone may enhance its anti-inflammatory effect [6]. The same group of authors reported that prenylated compounds with flavanone structure and hydroxyl substitution in C3 decreased antibacterial activity but had no effect on cytotoxic activity, while C8 prenylation can increase potent enzyme inhibitory activity, and this effect and C5′-prenylated chalcones with C6′-OH substitution have significant cytotoxic activity. Flavonoids can occur in free form, but in plants, they more commonly occur in conjugated form as flavonoid glycosides, which are conjugated by a bond between flavonoid aglycones and glycosyl groups. The glycosidic bond is located at position C3 or C7, and the carbohydrates are usually *L*-rhamnose, *D*-glucose, glucose–rhamnose, galactose, or arabinose [3]. The glycosylation of flavonoids can alter the biological activity of flavonoids, increase water solubility, reduce toxic effects and side effects, and improve specific targeting [7].

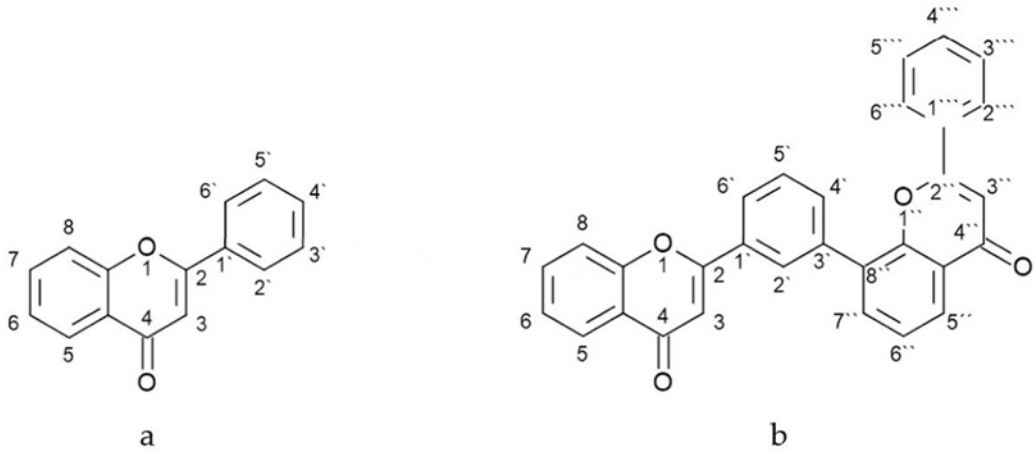

Figure 1. The basic structure of (**a**) flavonoids and (**b**) 3′-8″-biflavones.

Flavonoids can also occur in a dimeric form consisting of two monoflavonoid residues. The basic structure of the 3′-8″ dimer is presented in Figure 1b. To date, nearly 600 different biflavonoids have been described [8], but little is known about their biosynthesis and precise roles in plants. On the other hand, there is increasing evidence that flavonoid dimers are an effective new scaffold for drug discovery [8–13]. They have shown great potential as antimicrobial agents against viruses [14,15] and fungi [16], as well as in the treatment of neurodegenerative diseases [17] and cancers [18]. The precise mechanisms through which dimerization affects biological activity and whether this effect exists are not well understood. In this work, we aimed to investigate how the 3′-8″-dimerization affects the biological activity of flavonoids. To this end, we studied their scavenging activity (with DPPH), antifungal activity against mycotoxigenic fungi (*A. alternata*, *A. flavus*, *A. ochraceus*, *F. graminearum*, and *F. verticillioides*) (Figure 2), and inhibitory activity on enzymes (acetylcholinesterase, tyrosinase, α-amylase, and α-glucosidase) of five 3′-8″-biflavones (amentoflavone, bilobetin, ginkgetin, and isoginkgetin) and their monomeric subunits (apigenin, genkwanin, and acacetin).

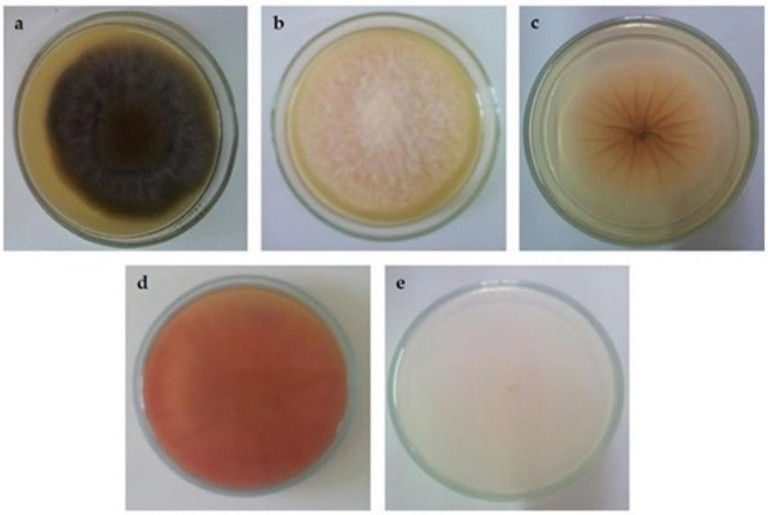

Figure 2. Photographs of the tested fungi without treatments: (**a**) *A. alternata*; (**b**) *A. flavus*; (**c**) *A. ochraceus*; (**d**) *F. graminearum*; (**e**) *F. verticillioides*.

2. Materials and Methods

2.1. Reagents and Standards

Amentoflavone (1) (CAS 1617-53-4), 4-nitrophenyl α-D-glucopyranoside (CAS 3767-28-0), Tris base (CAS 77-86-1), acetylcholinesterase from *Electrophorus electricus* (CAS 9000-81-1), 5,5′-dithiobis(2-nitrobenzoic acid) (CAS 69-78-3), 3,4-dihydroxy-L-phenylalanine (CAS 59-92-7), α-amylase from porcine pancreas (CAS 9000-90-2), 2,2-diphenyl-1-picrylhydrazyl (CAS 1896-66-4), 3,5-dinitrosalicylic acid (CAS 609-99-4), potassium sodium tartrate tetrahydrate (CAS 6381-59-5), starch (CAS 9005-84-9), tyrosinase from mushroom (CAS 9002-10-2), α-glucosidase from *Saccharomyces cerevisiae* (CAS 9001-42-7), 4-nitrophenyl α-D-glucopyranoside (CAS 3767-28-0), and RPMI-1640 medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bilobetin (2) (CAS 521-32-4), isoginkgetin (3) (CAS 548-19-6), ginkgetin (4) (CAS 481-46-9), sciadopitysin (5) (CAS 521-34-6), genkwanin (7) (CAS 437-64-9), and acacetin (8) (CAS 480-44-4) were obtained from PhytoLab (Vestenbergsgreuth, Germany). Sodium phosphate, monobasic monohydrate (CAS 10049-21-5), Sodium phosphate, dibasic heptahydrate (CAS 7782-85-6), and dimethyl sulfoxide (CAS 67-68-5) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Apigenin (6) (CAS 520-36-5) was purchased from Alfa Aesar (Ward Hill, MA, USA) and S-acetylthiocholine iodide (CAS 1866-15-5) was purchased from Biosynth (Bratislava, Slovakia). Methanol (CAS 67-56-1) was obtained from Kemika (Zagreb, Croatia) and sodium hydroxide (CAS 1310-73-2) was obtained from T.T.T. (Sveta Nedjelja, Croatia).

2.2. DPPH Scavenging Activity

DPPH scavenging activity was determined according to the method of Brand-Williams et al. [19]. In the DPPH assay, 20 µL of each standard (1 mg/mL) was mixed with 980 µL of 0.094 mM methanolic DPPH solution. After 1 h, absorbance at 515 nm was measured, and the results are presented as a percentage of DPPH radical inhibition.

2.3. Antifungal Activity

The antifungal activity of the tested standards was investigated according to the method described in [20]. Fungi that were used in this experiment are major producers of mycotoxins and food contaminants—*Alternaria alternata* (WT), *Aspergillus flavus* (NRRL 3251), *Aspergillus ochraceus* (CBS 589.68), *Fusarium graminearum* (CBS 110.250), and *Fusarium verticillioides* (CBS 119.825) (Figure 2). The compounds were tested at concentrations of 0.01, 0.1, 1, and 10 µg/mL, and the results are expressed as a percentage of fungal growth.

2.4. Enzyme Inhibition Activity

The acetylcholinesterase inhibition assay was performed using the reaction-based assay of Ellman [21] with modifications [22]. An enzyme solution (25 µL, 0.25 U/mL), Ellman's reagent (125 µL, 3 mM), and 50 µL of the tested compounds (100 µM) were mixed and preincubated for 15 min at room temperature. Then, 25 µL of S-acetylthiocholine iodide (15 mM) was added and incubated for 15 min at room temperature. The absorbance was measured at 405 nm using a plate reader. The blank sample was prepared in the same way with Tris-HCl buffer (50 mM, pH 8, 25 °C). The results are expressed as a percentage of inhibition.

The tyrosinase inhibition assay was performed according to Jakimiuk et al. [22], with slight modifications. The compounds (80 µL, 100 µM) were preincubated with 40 µL of a tyrosinase solution (250 U/mL) at room temperature for 10 min. Then, 80 µL of 3,4-dihydroxy-L-phenylalanine (3 mM) was added and incubated at room temperature for 10 min. The absorbance was measured at 492 nm in the plate reader. The blank was prepared using a PBS buffer (100 mM, pH 6.8, 25 °C). The results are expressed as a percentage of enzyme inhibition.

The α-amylase inhibition assay was performed according to Etsassala et al. [23], with slight modifications. In a microplate, 20 µL of each flavonoid (100 µM), 50 µL of the PBS buffer (100 mM, pH 6.8, 25 °C), and 10 µL of alpha-amylase (2 U/mL) were mixed and incubated at 37 °C for 20 min. Then, 10 µL of a 1% starch solution was added and incubated for the next 30 min. DNS reagent (100 µL) was added and boiled at 80 °C for 20 min. The absorbance was measured at 450 nm in the plate reader. The blank was prepared with PBS buffer (100 mM, pH 6.8, 25 °C). The results are expressed as a percentage of enzyme inhibition.

The α-glucosidase inhibition assay was performed according to Tiwari et al. [24], with slight modifications. Flavonoid solutions (100 µL, 100 µM) were incubated with 50 µL of α-glucosidase (1 U/mL) for 10 min at 37 °C. Then, 50 µL of 5 mM 4-nitrophenyl α-D-glucopyranoside was added. After 5 min, absorbance was measured at 405 nm in the plate reader. The blank was prepared with the PBS buffer (100 mM, pH 6.8, 25 °C). The results are expressed as a percentage of enzyme inhibition.

2.5. Statistical Analysis

All analyses were performed in at least triplicate, and the results are expressed as mean ± standard deviation (SD). All statistical analyses were performed using PAST software (version 4.13) [25]. One-way ANOVA and post hoc multiple mean comparisons (Tukey's HSD test) were performed, and differences between measurements were considered significant at $p < 0.05$.

3. Results

3.1. Chemical Formula of Investigated Standards

Here, we studied five 3′-8″-biflavones (amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin) and their monomeric subunits (apigenin, genkwanin, and acacetin), and their structures are shown in Figure 3. The number and position of methoxy and hydroxyl groups in their structures are summarized in Table 1.

As shown in Figure 3 and Table 1, all the dimers studied are dimers of the 3′-8″ type. Amentoflavone is a dimer of apigenin. The monomeric units of bilobetin are acacetin and apigenin, while isoginkgetin is an acacetin dimer. Ginkgetin consists of apigenin 4′,7-dimethyl ether and apigenin, whereas the monomeric subunits of sciadopitysin consist of apigenin 4′,7-dimethyl ether and acacetin. Unfortunately, apigenin 4′,7-dimethyl ether was not commercially available for inclusion in this study at the time the experiments were performed.

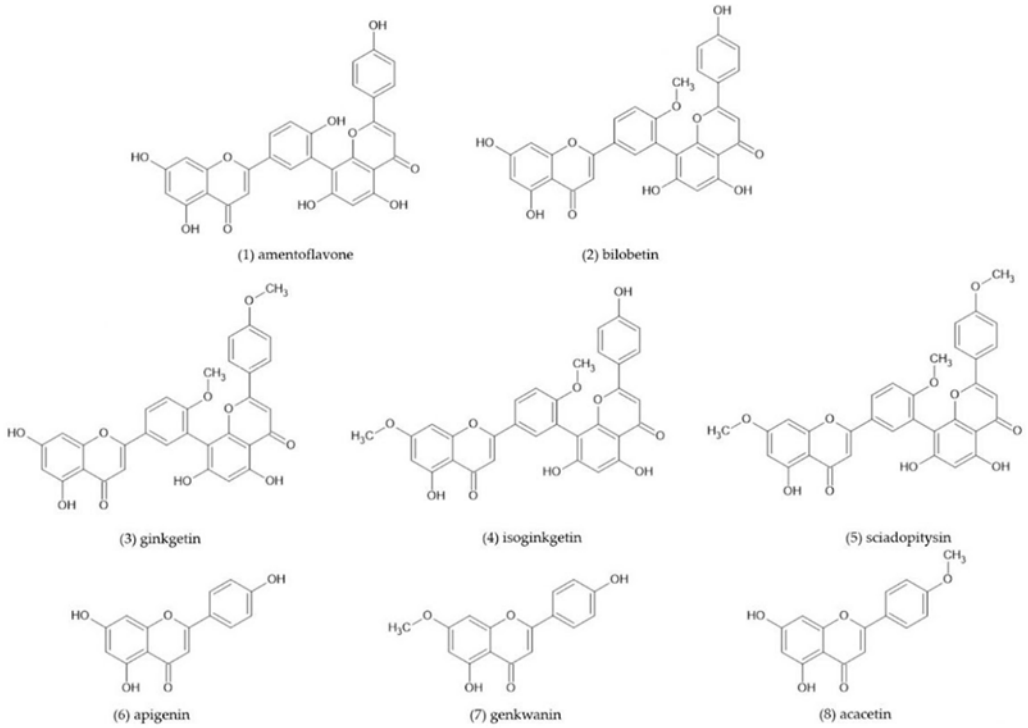

Figure 3. Chemical structure of investigated compounds.

Table 1. Summarized information about the number and position of hydroxy and methoxy groups of investigated compounds.

	Hydroxy		Methoxy		Dimer
	Number	**Position**	**Number**	**Position**	**Yes**
Amentoflavone	6	4′, 4‴, 5, 5″, 7, 7″	0	0	Yes
Bilobetin	5	4′, 5, 5″, 7, 7″	1	4‴	Yes
Ginkgetin	4	4‴, 5, 5″, 7″	2	4′, 7	Yes
Isoginkgetin	4	5, 5″, 7,7 ″	2	4′, 4‴	Yes
Sciadopitysin	3	5, 7	3	4′, 4‴, 7″	Yes
Apigenin	3	4′, 5, 7	0	0	No
Genkwanin	2	5, 4′	1	7	No
Acacetin	2	5, 7	1	4′	No

3.2. Antioxidant Activity

We measured antioxidant activity using the commonly used DPPH method, and the results are presented in Table 2.

Overall, all tested compounds showed very weak or no radical scavenging capacity, with no significant differences between them. The flavonoid quercetin, for example, showed 100% inhibition at the same concentration.

3.3. Antifungal Activity

In our experiment, we compared the antifungal activity against five fungi (Figure 2) at different concentrations and recorded the results at two wavelengths (Table 3).

Table 2. Radical scavenging activity of compounds at concentration 1 mg/mL.

	DPPH Radical Scavenging (% Inhibition)
Amentoflavone	4.60 ± 2.00 [a]
Bilobetin	4.56 ± 0.97 [a]
Ginkgetin	2.62 ± 1.41 [a]
Isoginkgetin	2.99 ± 1.55 [a]
Sciadopitysin	2.02 ± 00 [a]
Apigenin	3.18 ± 1.28 [a]
Acacetin	1.93 ± 1.27 [a]
Genkwanin	3.01 ± 0.61 [a]

Parameters sharing the same letter do not differ significantly at $p > 0.05$.

Table 3. Growth percentages of tested fungi grown under different flavonoid concentrations.

		A. alternata			
	Abs	**0.01 µg/mL**	**0.1 µg/mL**	**1 µg/mL**	**10 µg/mL**
Amentoflavone	**405**	96.39 ± 26.85	92.33 ± 37.88	83.00 ± 23.98	104.85 ± 10.71
	450	102.05 ± 27.28	93.65 ± 44.91	82.19 ± 28.09	70.11 ± 10.10
Bilobetin	**405**	79.53 ± 6.55	103.26 ± 33.66	67.67 ± 17.69	115.77 ± 21.30
	450	80.21 ± 6.82	106.76 ± 36.96	67.66 ± 21.07	91.20 ± 23.80
Ginkgetin	**405**	107.49 ± 31.25	88.63 ± 9.45	69.78 ± 6.57	98.68 ± 7.38
	450	113.31 ± 39.85	87.92 ± 9.65	68.07 ± 10.56	64.18 ± 13.33
Isoginkgetin	**405**	102.03 ± 10.01	103.96 ± 24.38	91.10 ± 13.17	96.92 ± 20.86
	450	106.96 ± 13.15	104.30 ± 25.93	93.04 ± 16.55	89.97 ± 40.77
Sciadopitysin	**405**	97.27 ± 19.89	92.51 ± 19.49	86.52 ± 21.03	85.64 ± 27.76
	450	101.64 ± 20.87	93.45 ± 22.60	87.92 ± 23.46	74.21 ± 30.19
Apigenin	**405**	106.96 ± 13.80	107.82 ± 30.62	105.76 ± 19.05	85.14 ± 21.09
	450	106.96 ± 13.80	107.82 ± 30.62	105.76 ± 19.05	85.14 ± 21.09
Genkwanin	**405**	133.42 ± 15.95	98.88 ± 29.52	103.35 ± 20.77	100.95 ± 24.86
	450	133.42 ± 15.95	98.88 ± 29.52	103.35 ± 20.77	100.95 ± 24.86
		A. flavus			
Amentoflavone	**405**	95.77 ± 14.78	80.90 ± 21.43	101.51 ± 20.18	87.77 ± 5.73
	450	98.44 ± 14.30	79.87 ± 21.30	100.00 ± 20.84	66.29 ± 5.07
Bilobetin	**405**	83.73 ± 23.45	77.05 ± 18.09	80.81 ± 15.21	85.70 ± 11.09
	450	84.37 ± 24.04	75.96 ± 18.67	78.11 ± 12.15	70.10 ± 12.13
Ginkgetin	**405**	123.49 ± 16.01	85.70 ± 26.01	67.36 ± 18.63	86.64 ± 13.77
	450	126.97 ± 13.37	87.20 ± 26.62	64.53 ± 18.92	68.83 ± 16.35
Isoginkgetin	**405**	87.86 ± 14.00	100.47 ± 14.07	85.79 ± 23.79	83.16 ± 10.27
	450	86.61 ± 16.26	100.88 ± 12.10	86.22 ± 24.90	70.69 ± 12.55
Sciadopitysin	**405**	83.73 ± 23.45	77.05 ± 18.09	80.81 ± 15.21	85.70 ± 11.09
	450	101.37 ± 15.36	70.59 ± 14.14	76.06 ± 18.01	64.92 ± 6.07
Apigenin	**405**	122.77 ± 0.68	98.32 ± 28.87	59.98 ± 12.89	116.06 ± 28.23
	450	127.93 ± 6.75	101.69 ± 32.22	57.88 ± 14.82	109.10 ± 26.78
Genkwanin	**405**	92.81 ± 12.75	108.39 ± 27.25	115.42 ± 26.45	134.51 ± 28.13
	450	92.15 ± 14.70	109.98 ± 28.23	115.97 ± 27.17	132.96 ± 36.58

Table 3. *Cont.*

			A. ochraceus		
Amentoflavone	**405**	90.39 ± 24.20	71.37 ± 14.29	70.17 ± 11.90	111.01 ± 9.84
	450	92.27 ± 23.16	73.99 ± 12.64	69.64 ± 13.19	74.65 ± 2.88
Bilobetin	**405**	82.18 ± 4.95	67.57 ± 18.80	85.19 ± 12.11	131.63 ± 19.62
	450	80.96 ± 4.05	68.77 ± 19.37	87.49 ± 12.65	111.86 ± 24.49
Ginkgetin	**405**	96.20 ± 43.63	80.78 ± 22.13	80.38 ± 25.30	117.02 ± 9.44
	450	102.50 ± 45.50	82.92 ± 19.64	81.39 ± 23.17	79.43 ± 14.64
Isoginkgetin	**405**	75.38 ± 10.93	82.78 ± 18.20	83.98 ± 9.58	101.40 ± 14.93
	450	80.74 ± 10.97	84.66 ± 18.28	86.40 ± 8.45	90.32 ± 18.62
Sciadopitysin	**405**	84.58 ± 19.27	75.98 ± 10.35	75.98 ± 12.08	101.40 ± 13.16
	450	84.87 ± 19.79	74.43 ± 9.77	78.13 ± 14.20	86.62 ± 15.69
Apigenin	**405**	75.98 ± 30.47	67.60 ± 18.56	66.20 ± 14.16	54.26 ± 8.16
	450	78.24 ± 31.73	65.65 ± 16.83	67.31 ± 14.24	61.39 ± 24.44
Genkwanin	**405**	77.38 ± 26.74	59.56 ± 26.28	69.34 ± 15.73	116.51 ± 21.53
	450	78.06 ± 27.16	58.80 ± 25.89	67.31 ± 14.62	83.06 ± 9.30
			F. graminearum		
Amentoflavone	**405**	67.99 ± 20.62	68.89 ± 19.25	47.01 ± 7.74	83.00 ± 15.23
	450	58.20 ± 22.27	63.86 ± 29.56	38.72 ± 10.99	61.55 ± 18.03
Bilobetin	**405**	58.22 ± 6.52	76.70 ± 15.94	64.49 ± 20.76	90.91 ± 27.33
	450	55.16 ± 10.09	71.18 ± 18.64	62.19 ± 23.21	76.45 ± 28.66
Ginkgetin	**405**	54.99 ± 18.02	63.59 ± 12.16	57.59 ± 20.08	89.51 ± 21.64
	450	44.71 ± 23.54	53.71 ± 16.45	55.51 ± 25.52	67.97 ± 28.65
Isoginkgetin	**405**	48.48 ± 7.01	69.39 ± 10.40	61.29 ± 10.61	65.89 ± 14.61
	450	35.98 ± 12.50	55.25 ± 18.99	58.85 ± 12.44	57.43 ± 19.33
Sciadopitysin	**405**	52.62 ± 8.64	72.30 ± 20.12	55.82 ± 4.01	80.20 ± 25.92
	450	53.28 ± 15.54	68.23 ± 22.74	53.79 ± 8.48	69.00 ± 34.25
Apigenin	**405**	81.36 ± 22.43	79.16 ± 27.67	83.51 ± 26.00	92.09 ± 10.01
	450	78.58 ± 37.50	83.23 ± 43.84	74.05 ± 26.50	62.75 ± 12.56
Genkwanin	**405**	77.92 ± 22.72	74.43 ± 18.08	84.51 ± 19.32	114.73 ± 31.86
	450	70.70 ± 21.95	67.87 ± 26.52	83.05 ± 28.45	105.83 ± 29.53
			F. verticillioides		
Amentoflavone	**405**	104.84 ± 5.45	95.39 ± 19.55	102.31 ± 5.71	82.67 ± 6.20
	450	109.03 ± 7.77	100.16 ± 23.97	104.59 ± 5.74	78.16 ± 8.28
Bilobetin	**405**	103.38 ± 12.66	101.63 ± 9.10	111.53 ± 13.48	70.16 ± 4.84
	450	105.90 ± 13.52	105.53 ± 9.75	112.96 ± 15.25	66.31 ± 6.04
Ginkgetin	**405**	103.26 ± 11.04	113.67 ± 19.59	123.12 ± 15.76	80.08 ± 6.72
	450	109.28 ± 12.94	120.90 ± 23.15	129.52 ± 17.86	76.10 ± 7.43
Isoginkgetin	**405**	110.97 ± 12.60	101.91 ± 7.77	117.78 ± 6.58	84.36 ± 8.42
	450	115.71 ± 16.08	106.53 ± 11.35	122.21 ± 7.83	83.04 ± 7.26
Sciadopitysin	**405**	107.88 ± 20.54	97.02 ± 5.27	102.53 ± 18.36	71.70 ± 8.89
	450	114.34 ± 24.68	100.53 ± 6.77	107.40 ± 20.60	71.35 ± 10.72
Apigenin	**405**	122.94 ± 11.65	102.53 ± 8.07	121.55 ± 16.24	88.68 ± 9.07
	450	132.06 ± 11.84	106.53 ± 9.13	128.44 ± 19.07	93.27 ± 10.02
Genkwanin	**405**	98.07 ± 25.09	107.89 ± 19.90	105.42 ± 5.58	105.48 ± 3.18
	450	114.35 ± 24.59	113.63 ± 21.19	108.34 ± 6.71	103.72 ± 7.75

As can be seen from the table, the antifungal activity depends on both the fungi used and the tested concentration. At the lowest concentration, 0.01 µg/mL, all the tested

compounds showed significant inhibition only against *F. graminearum*, and the biflavonoids showed higher inhibition than the tested monomers. In the case of *F. verticillioides*, all biflavonoids and apigenin showed inhibition only at a concentration of 10 µg/mL, whereas at lower concentrations, they showed no inhibition or even slightly growth-promoting effects. In contrast, the antifungal activity against *A. ochraceus* was higher for all biflavonoids and genkwanin at lower concentrations and decreased with the increase in concentration. All biflavonoids showed antifungal activity against A. flavus, and the activity increased with concentration for most biflavonoids. Apigenin showed antifungal activity against A. flavus only at a concentration of 1 µg/mL, while genkwanin showed dose-dependent stimulatory effects on this fungus. In the case of *A. alternata*, antifungal activity was strongly concentration-dependent, but in general, biflavonoids showed weak activities, while apigenin and genkwanin showed no antifungal activity.

3.4. Enzyme Inhibition Activity

Enzyme inhibition activities of the compounds at 100 µM are shown in Figure 4. We measured inhibitory activities against four enzymes—acetylcholinesterase, tyrosinase, α-amylase, and α-glucosidase. Ginkgetin (26.24 ± 1.71%) and isoginkgetin (25.37 ± 0.66%) inhibited acetylcholinesterase significantly more than the other compounds tested. Tyrosinase inhibition activity was higher for the monomeric flavonoids acacetin (16.68 ± 0.67%), apigenin (15.85 ± 0.34%), and genkwanin (13.12 ± 1.65%) than for the dimeric flavonoids. Ginkgetin showed no inhibition against tyrosinase. The inhibition against α-amylase was highest with amentoflavone (56.26 ± 1.32%), followed by isoginkgetin (42.00 ± 3.68%) and bilobetin (32.76 ± 1.72%). Amentoflavone showed complete inhibition against α-glucosidase (98.17 ± 0.56%), and the other biflavonoids tested, ginkgetin (85.36 ± 1.06%), isoginkgetin (78.42 ± 4.15%), sciadopitysin (60.16 ± 2.90%), and bilobetin (49.36 ± 3.10%), also showed high inhibition compared with the monomeric flavonoids.

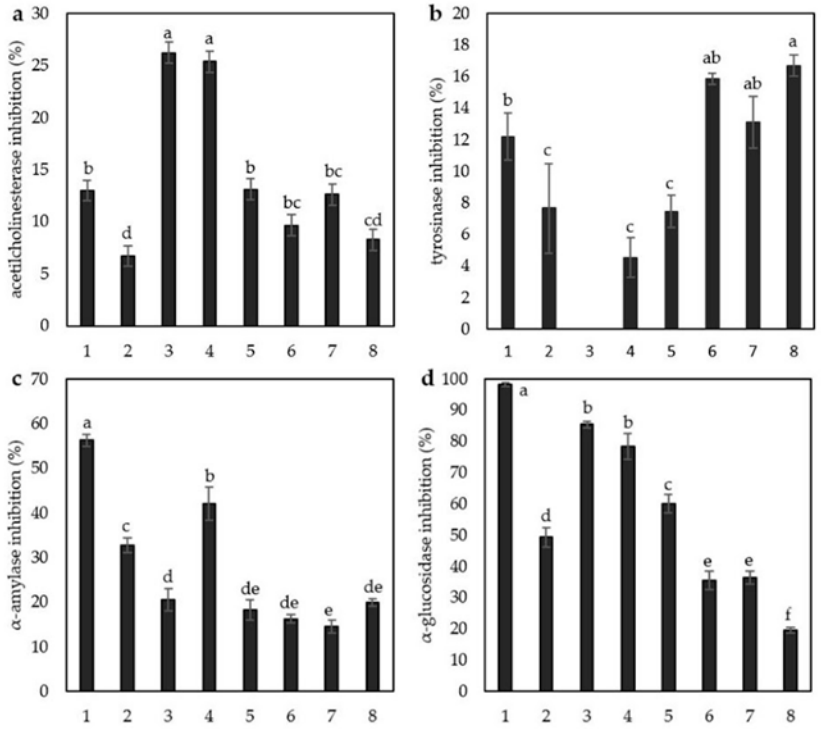

Figure 4. Enzyme inhibition activity of (1) amentoflavone, (2) bilobetin, (3) ginkgetin, (4) isoginkgetin, (5) sciadopitysin, (6) apigenin, (7) genkwanin, and (8) acacetin against (**a**) acetylcholinesterase, (**b**) tyrosinase, (**c**) α-amylase, and (**d**) α-glucosidase at a concentration of 100 µM. Values with different letters differ significantly at $p < 0.05$.

4. Discussion

Flavonoids are often considered antioxidants, but in reality, their antioxidant activity is highly dependent on the structure of individual flavonoids [26]. This is also evident from our results showing that flavones and biflavones have weak radical scavenging activity using one of the most commonly used methods for measuring antioxidant activity, DPPH. Similar to the results of our experiments, Kang et al. [27] revealed that none of the five biflavones also tested in our study showed radical scavenging activity up to 100 µM when measured with 1,1-diphenyl-2-picrylhydrazyl (DPPH). We tested even higher concentrations and observed no significant activity. This suggests that biflavones do not act as antioxidants or radical scavengers, at least in a cell-free system. Research findings also show that the biflavones amentoflavone, bilobetin, ginkgetin, and sciadopitysin are the weakest antioxidants among the 30 compounds isolated from ginkgo tested in myelomonocytic HL-60 cells [28]. However, there are some conflicting data on the antioxidant activity of biflavones. For example, Li et al. [29] compared the antioxidant activity of acacetin and its 3′,8″ dimer isoginkgetin using three methods, the O^{2-} scavenging assay, the Cu^{2+} reducing assay, and the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay, and reported that the 3′,8″-dimerization increased the antioxidant capacity of flavonoids. From all these findings, we can conclude that the antioxidant activity of biflavones is still very poorly understood, and further studies should clarify their activity as antioxidants.

Monomeric flavonoids have been shown to be effective antifungal agents against a variety of pathogenic organisms through mechanisms that include the disruption of the plasma membrane; the induction of mitochondrial dysfunction; and the inhibition of cell wall formation, cell division, RNA and protein synthesis, and efflux-mediated pumping [30]. Herein, we tested compounds for their potential inhibition against five mycotoxigenic fungi that are perhaps the most important pathogens of global concern in the context of food safety. They can affect the quality and quantity of marketable products by damaging foods such as corn, wheat, and peanuts and producing mycotoxin metabolites that can be carcinogenic and affect human and animal health [31]. According to our results, antifungal activity depends on both the fungi used and the tested concentration. The genus *Fusarium* generates a number of mycotoxins that can cause acute or chronic disease and, in some cases, death [32]. At the lowest concentration, 0.01 µg/mL, all the tested compounds showed significant inhibition against *F. graminearum*, and biflavonoids showed higher inhibition than the monomers analyzed. When we directly compared amentoflavone with its monomeric subunits apigenin, we found that amentoflavone had a stronger inhibition against *F. graminearum*, which may indicate that the 3′-8″-dimerization increases the antifungal activity against this fungus. In the case of *F. verticillioides*, all biflavonoids and apigenin showed inhibition at a higher concentration (10 µg/mL). Krauze-Baranowska and Wiwart [16] investigated the antifungal activity of amentoflavone, bilobetin, ginkgetin, and sciadopitysin against *F. culmorum*, and at a concentration of 100 µM/mL, bilobetin, ginkgetin, and sciadopitysin showed 100% inhibition. It has also been reported that the extracts of *Hypericum triquetrifolium* have antifungal activity against *Fusarium* sp. [33], and *Hypericum* sp. are known to contain biflavonoids [34]. Taken together, these findings indicate that the biflavonoids tested here can be potential inhibitors of *Fusarium* sp. Other mycotoxigenic fungi we tested belong to *Aspergillus* sp., namely *A. flavus* and *A. ochraceus*. All the tested biflavonoids, as well as genkwanin, showed antifungal activity against *A. ochraceus*, which was higher at lower concentrations and decreased with the increase in concentration, whereas for *A. flavus*, activity increased with concentration for most biflavonoids. Previously, isoginkgetin was reported to have inhibitory activity against *A. fumigatus* [35]. Gonçalez et al. [36] reported in their study that amentoflavone inhibited the production of aflatoxin B1 and B2 by *A. flavus* but did not inhibit fungal growth at the concentration tested. They indicated that biflavonoids may be effective agents for controlling aflatoxin production without inhibiting growth. All the compounds we tested here showed weak or no inhibition against *A. alternata* at concentrations up to 10 µg/mL.

Krauze-Baranowska and Wiwart [16] investigated antifungal activity at a higher concentration of 100 µM and showed that ginkgetin had 100% inhibition against *A. alternata*, while the inhibition of amentoflavone, bilobetin, and sciadopitysin was 54%, 80%, and 59%, respectively.

For the enzyme inhibitory activities of our compounds, we selected four enzymes that are involved in important metabolic functions. Acetylcholinesterase (AChE) inhibitors are widely used for the symptomatic treatment of Alzheimer's disease and other dementias because the inhibition of AChE slows the hydrolysis of acetylcholine and increases choline levels, which improves cognitive function. Monomeric flavonoids have been recognized as AchE inhibitors for several decades [37]. According to our results, at a concentration of 100 µM, ginkgetin and isoginkgetin had significantly higher inhibitory effects on acetylcholinesterase than the other compounds analyzed. These results are not surprising because ginkgetin and isoginkgetin are characteristic compounds of *Ginkgo biloba* L. [9,28,38], a plant used to treat cognitive disorders, and ginkgetin is known to be a potential neuprotectant [17]. The inhibition of tyrosinase is related to the potential use of compounds to reduce melanogenesis activity and alleviate hyperpigmentation [39]. According to our results, all three monomeric flavonoids, i.e., apigenin, genkwanin, and acacetin, showed significantly higher inhibitory activity than the biflavones studied. Among the biflavonoids, amentoflavone showed higher inhibitory activity. According to the molecular docking study by Ogunwa [40], amentoflavone has moderate tyrosinase inhibitory potential, which is also evident in our study.

We also investigated the inhibitory activity against α-amylase and α-glucosidase, the two enzymes involved in carbohydrate metabolism. The inhibition of these enzymes may help regulate blood glucose levels and prevent/control diabetes mellitus [23]. According to our results, amentoflavone is the best α-amylase inhibitor, followed by isoginkgetin. Ametoflavone has already been recognized as a potent α-amylase inhibitor, and its possible mechanism of action involves the occupation of the catalytic site and other regions of the enzyme as well as the inhibition of the substrate's access and binding [40]. Peterson et al. [41] studied bilobetin, isoginkgetin, ginkgetin, and sciadopitysin isolated from a ginkgo for their α-amylase inhibitory activity. They found that bilobetin had no clear inhibitory effect, whereas, in the other components, the inhibitory effect decreased with the increase in concentration, and it was most pronounced for sciadopitysin, which they characterized as an α-amylase activator rather than an inhibitor. These results are consistent with our results where sciadopitysin showed the least inhibitory effect on α-amylase. In the case of α-glucosidase, amentoflavone showed complete inhibition under the concentration tested, followed by isoginkgetin and ginkgetin. Amentoflavone is already known to be a potent α-glucosidase inhibitor. Swargiary et al. [42] investigated α-glucosidase inhibitory activity via the molecular docking of 155 different phenolic compounds and found that amentoflavone had the strongest binding affinity with α-glucosidase, much stronger than the reference acarbose. Similarly, Li et al. [43] investigated the inhibitory effects of amentoflavone and monomeric apigenin on α-glucosidase and reported a stronger inhibitory effect of amentoflavone, which is also reflected in our results. Flavonoids are noncompetitive inhibitors of α-glucosidase and have shown synergistic inhibitory effects with acarbose [43], an α-glucosidase inhibitor used in conjunction with diet and exercise to control blood glucose levels in patients with type 2 diabetes mellitus.

5. Conclusions

In the present study, we evaluated the radical scavenging activity (with DPPH), antifungal activity against mycotoxigenic fungi (*A. alternata*, *A. flavus*, *A. ochraceus*, *F. graminearum*, and *F. verticillioides*), and enzyme inhibition (acetylcholinesterase, tyrosinase, α-amylase, and α-glucosidase) of five 3′-8″-biflavones (amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin) and their monomeric subunits (apigenin, genkwanin, and acacetin). All the tested compounds showed weak radical scavenging activity. The antifungal activity strongly depends on the fungi used and the concentration. At some concentrations, we

even detected growth-promoting activity, as in the case of *F. verticillioides*. At the lowest concentration, 0.01 µg/mL, all the analyzed compounds showed significant inhibition against *F. graminearum*, and the biflavonoids showed higher inhibition than the tested monomers. The antifungal activity against *A. ochraceus* was higher for all biflavonoids and genkwanin at lower concentrations and decreased with the increase in concentration, while all biflavonoids showed antifungal activity against *A. flavus*, and the activity increased with concentration for most biflavonoids. Biflavonoids showed weak activities against *A. alterata*, while apigenin and genkwanin showed no antifungal activity. Isoginkgetin and ginkgetin showed the highest inhibition against acetylcholinesterase, while monomeric compounds showed higher inhibitory activity against tyrosinase. Amentoflavone proved to be a potent inhibitor of α-amylase and α-glucosidase, and the 3′-8″-biflavones tested showed higher inhibitory activity against α-glucosidase than their monomeric subunits. From all these results, we can conclude that the 3′-8″-dimerization affects the biological activity, such as the antifungal activity against *F. graminearum*, where the biflavonoids showed higher inhibitory activity than the tested monomers. Also, the inhibitory activity against α-amylase and α-glucosidase is enhanced by 3′-8″-dimerization but is also influenced by the number of methoxy and hydroxyl groups.

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5.1.2. Tissue-Specific Profiling of Biflavonoids in Ginkgo (*Ginkgo biloba* L.)

plants

MDPI

Communication

Tissue-Specific Profiling of Biflavonoids in Ginkgo (*Ginkgo biloba* L.)

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Abstract: Biflavonoids are flavonoid dimers that are much less studied than monomeric flavonoids. Their precise distribution among plants and their role in plants is still unknown. Here, we have developed a HPLC-DAD method that allows us to separate and simultaneously determine the five major biflavonoids (amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin) in ginkgo (*Ginkgo biloba* L.). We performed tissue-specific profiling of biflavonoids in ten different plant parts: tree bark, twigs bark, twigs without bark, buds, leaf petioles, leaf blades, seed stalks, sarcotesta, nutshells, and kernels. We did not detect biflavonoids in plant parts not in direct contact with the environment (twigs without bark, nutshells, and kernels). We found the highest total biflavonoids content in leaves, where sciadopitysin was predominant. In contrast, in the bark, amentoflavone was the predominant biflavonoid, suggesting that more methylated biflavonoids accumulate in leaves and seeds. This is probably related to their biological function, which remains to be determined.

Keywords: amentoflavone; bilobetin; ginkgetin; isoginkgetin; sciadopitysin; ginkgo; tissue-specific profiling; HPLC-DAD

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1. Introduction

Flavonoids are a large, and to date, most studied group of plant metabolites [1]. Flavonoids have a 15 carbon- atom flavone skeleton, C6-C3-C6, with two benzene rings (A and B) joined by a trinuclear pyran ring (C). The position of the B catechol ring on the C pyran ring, as well as the number and position of hydroxyl groups on the catechol group of the B ring, have a major influence on the chemical properties and biological activity of flavonoids [1–3]. In addition, flavonoids can be conjugated, glycosylated, or methylated, which also affects their biological properties and function in plants [1]. Flavonoid dimers, known as biflavonoids, consist of two monomeric flavonoids via a direct link or a linear linker. According to He et al. [4], nearly 600 different biflavonoids are known in angiosperms, ferns, gymnosperms, and bryophytes. Most of them are found in plants used in traditional medicine and are considered important factors in the health benefits of these plants [3,5,6]. However, the role of biflavonoids in plants is poorly studied. Based on their biological activity and localization in leaves [7,8], there are indications that they may be involved in protecting plants from pests and predators and in photosynthesis regulation [9].

A plant in which various biflavonoids have been detected is the ginkgo (*Ginkgo biloba* L.), also called maidenhair tree [3,10]. It is a deciduous gymnosperm tree (family Ginkgoaceae) native to China and has been planted in Chinese and Japanese temple gardens since ancient times, but is now found as an ornamental tree in many parts of the world [11]. Its use in traditional Chinese medicine is well known, but today its extracts are widely used worldwide for the treatment of cognitive complaints [12]. Various specialized metabolites have been detected in ginkgo plant samples and extracts, including 110 different flavonoids, 13 of which are biflavonoids [3,10]. Although the presence of other biflavonoids has also been reported, the most commonly reported biflavonoids include amentoflavone, bilobetin,

ginkgetin, isoginkgetin, and sciadopitysin [3]. These are all 3′, 8″- biflavones whose basic structure is shown in Figure 1.

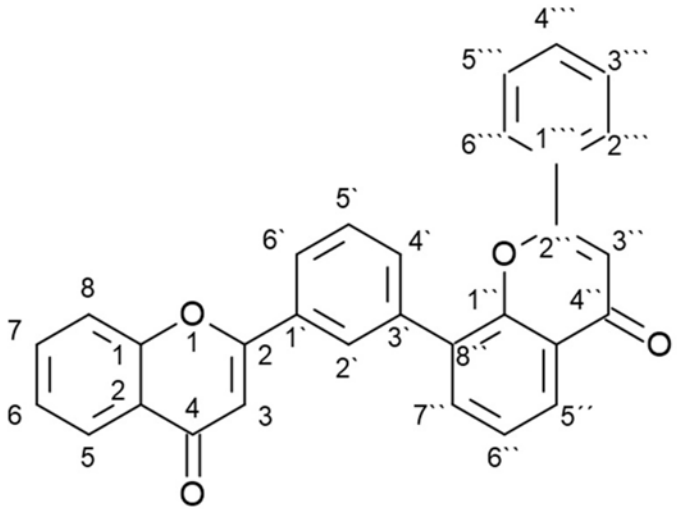


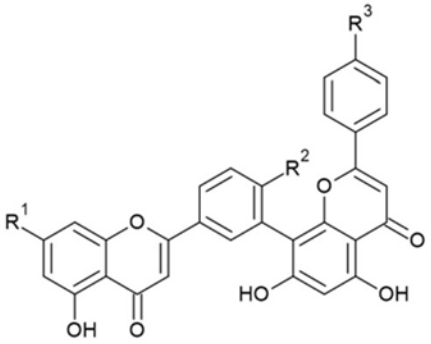
Figure 1. Basic structure of 3′, 8″- biflavones.

The flavonoids in ginkgo are being researched by many scientists. However, most studies focus on monomeric flavonoids and biflavonoids are often neglected [13–15], e.g., in the review article by Liu et al. [16], which focuses on the advances in chemical analysis and quality control of flavonoids in ginkgo, the presence of biflavonoids is hardly mentioned. To fill this gap in our knowledge of ginkgo flavonoids, the aim of the present study was to establish a tissue-specific profile of biflavonoids in ginkgo. To this end, we developed the HPLC-DAD method that allowed us to simultaneously determine five biflavonoids, amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin. We determined their content in ten different ginkgo plant parts and discussed their presence in relation to possible physiological functions.

2. Results

2.1. Chemical Differences and Method for Biflavonoids Analysis

All five biflavonoids analyzed (Figure 2) belong to the amentoflavone type, in which two monomeric units are joined at positions 3′, 8″ (Figure 1). Amentoflavone consists of two apigenin monomers and other biflavonoids in ginkgo are considered derivatives of amentoflavone.



	R¹	R²	R³
amentoflavone	OH	OH	OH
bilobetin	OH	OMe	OH
isoginkgetin	OH	OMe	OMe
ginkgetin	OMe	OMe	OH
sciadopitysin	OMe	OMe	OMe

Figure 2. Chemical structure of main biflavonoids in ginkgo.

Bilobetin has a methoxy group at the C-4′ position. Isoginkgetin and ginkgetin have two methoxy groups–isoginkgetin at the C-4′ and C-4‴ positions and ginkgetin at C-7 and

C-4′. Sciadopitysin has three methoxy groups at C atom positions C-7, C-4′ and C-4‴. The presence of methoxy groups affects the chemical properties of the analyzed compounds and their polarity, which allows us to separate the compounds using reverse phase C 18 HPLC silica columns (Figure 3).

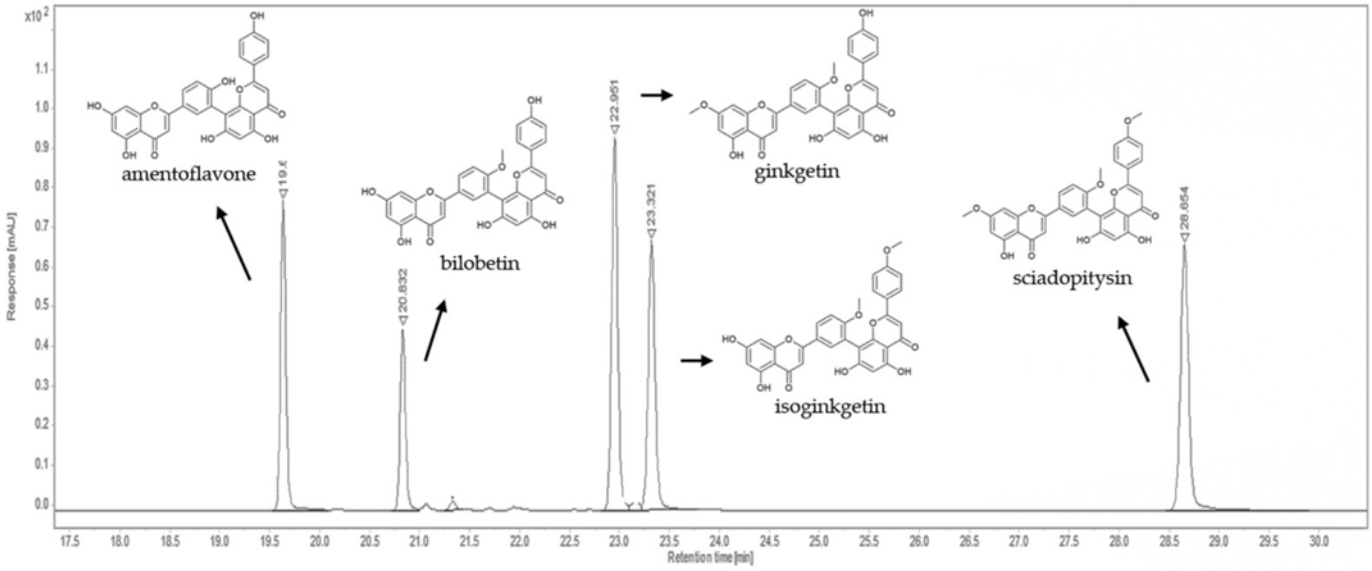


Figure 3. Representative chromatogram of five biflavones recorded at 330 nm.

Under the proposed instrumental conditions, the biflavonoids eluted at 19.6 min (amentoflavone), at 20.8 min (bilobetin), at 23.0 min (ginkgetin), at 23.3 min (isoginkgetin), and at 28.7 min (sciadopitysin), as shown in Figure 3 and Table 1. Identification of each compound was performed by comparing the retention times and UV spectra of the analytes with those of the standard solution. According to the criteria of Commission Decision (EC) No. 657/2002 on the performance of analytical methods [17], the relative retention time of the determined compound in the sample solution had to be equal to that in the standard solution with a tolerance of ±2.5%. Quantification was performed using an external standard calibration and the amount of biflavonoids in the plant samples was expressed in μg/g dry weight (dw).

Table 1. Retention times and UV spectrum data for analyzed biflavonoids.

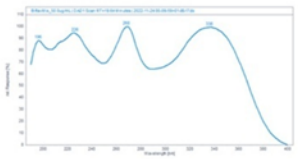
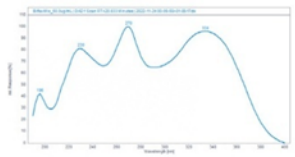
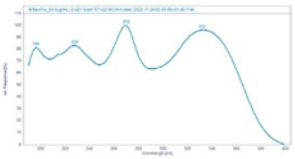
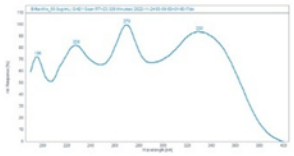
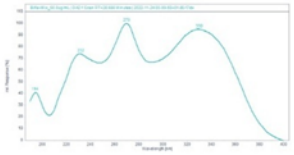
Compound	Retention Time (min)	Characteristic UV Spectrum	Maximum UV Apsorption (nm)
Amentoflavone	19.634		196, 226, 268, 336
Bilobetin	20.832		196, 230, 270, 334

Table 1. *Cont.*

Compound	Retention Time (min)	Characteristic UV Spectrum	Maximum UV Apsorption (nm)
Ginkgetin	22.951		196, 228, 270, 332
Isoginkgetin	23.321		196, 228, 270, 330
Sciadopitysin	28.654		194, 232, 270, 330

The limit of detection (LOD) and limit of quantification (LOQ) were estimated using the signal-to-noise (S/N) approach, calculated for the sample extract at the targeted level of 0.3 µg/mL for LOD and 1.0 µg/mL for LOQ, for each compound. The obtained S/N values were regarded as acceptable if ≥3 and ≥10, respectively. The achieved R^2 of all calibration curves was greater than 0.99, and the targeted LOD and LOQ values for all five compounds (0.3 µg/mL and 1.0 µg/mL, respectively) were evaluated as acceptable, as satisfying the requirements of sufficient S/N ratios (Table 2).

Table 2. Curve equation, R^2, LOD and LOQ for analyzed biflavonoids.

Analyte	Wavelength nm	Curve Equation	R^2	LOD µg/mL	LOQ µg/mL
Amentoflavone	330	y = 36.7275x − 51.0679	0.99504	0.30	1.0
Bilobetin	330	y = 23.2259x − 33.033	0.99758	0.30	1.0
Ginkgetin	330	y = 54.6868x − 74.7767	0.99765	0.30	1.0
Isoginkgetin	330	y = 44.1283x − 51.4203	0.99775	0.30	1.0
Sciadopitysin	330	y = 49.3575x − 59.0710	0.99806	0.30	1.0

2.2. Tissue-Specific Biflavonoids Profiling

To establish a tissue-specific biflavone profile, we separated the different ginkgo plant parts as shown in Figure 4. Various names have been used for the ginkgo seeds, which are sometimes called fruits, although the ginkgo belongs to the gymnosperms and produces seeds. The fleshy part is the seed coat or sarcotesta.

We can divide the analyzed plant parts into parts that are in direct contact with the environment, such as leaves (petiole and leaf blade), bark, twig bark, bud, seed petiole and sarcotesta, and parts that are not in direct contact with the environment, such as twig without bark, nutshell, and kernel. We did not detect biflavonoids in the parts that are not in direct contact with the environment.

Figure 5 shows the biflavonoid profile of tree and twig barks and buds. In tree bark, we find only amentoflavone at a concentration of 63.30 ± 4.60 µg/g dw. Amentoflavone was also the most abundant biflavonoid in twig bark and buds with a concentration of 75.70 ± 6.80 µg/g dw and 38.82 ± 1.55 µg/g dw, respectively. Other biflavonoids were also present in the twig bark: bilobetin in concentration 32.53 ± 2.30 µg/g dw, ginkgetin 33.79 ± 2.80 µg/g dw, isoginkgetin 29.49 ± 3.23 µg/g dw and sciadopitysin

41.57 ± 4.64 µg/g dw. In buds, we could not detect bilobetin, and ginketin, isoginkgetin, and sciadopitysin were found at concentrations of 13.79 ± 1.00 µg/g dw, 5.47 ± 1.03 µg/g dw, and 10.96 ± 3.43 µg/g dw, respectively.

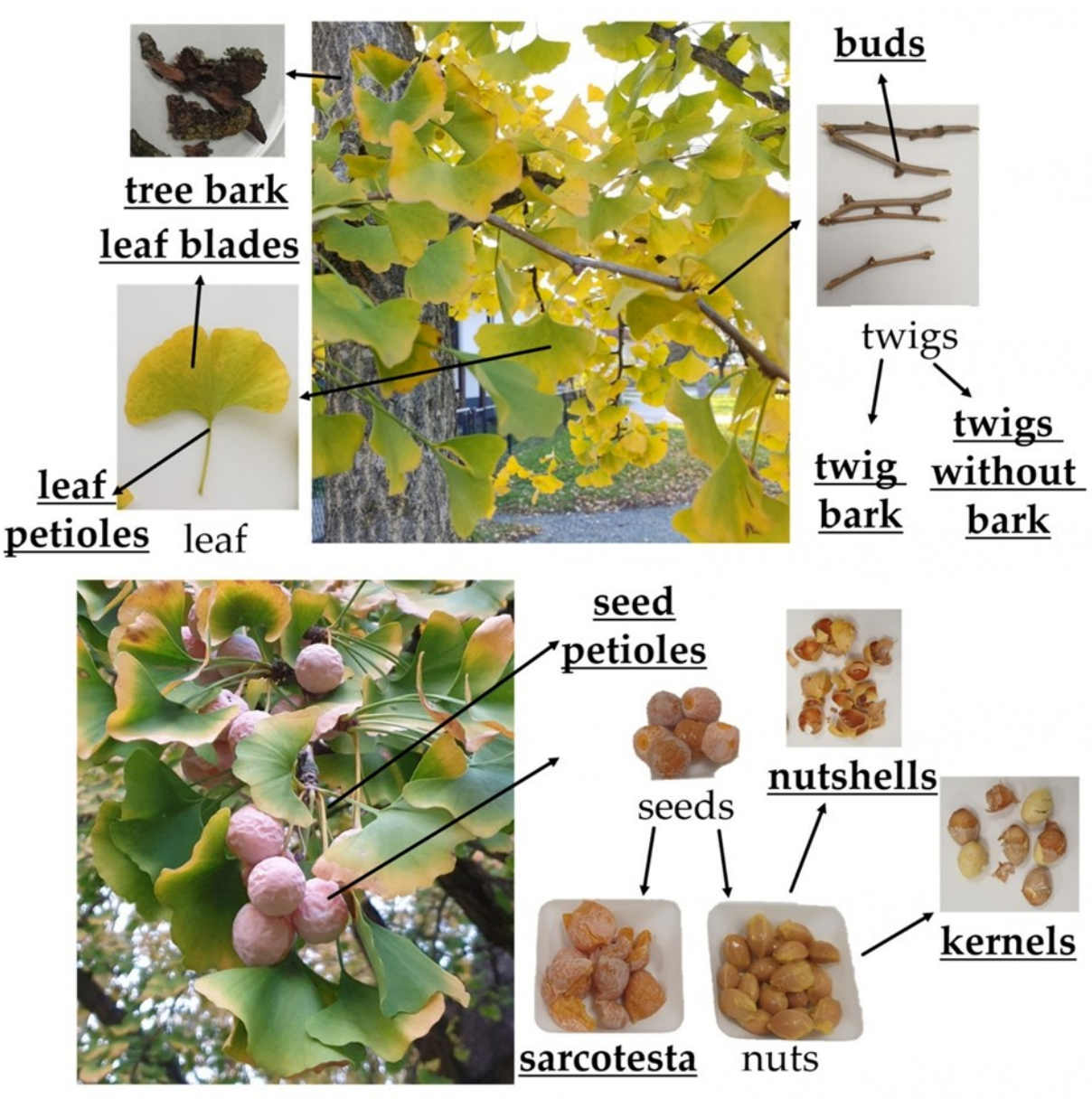


Figure 4. Ginkgo plant parts. Analyzed parts are bold and underlined.

We found the highest content of biflavonoids in the leaf petioles and blades (Figure 6). In contrast to the barks, amentoflavones were the least abundant biflavonoids in the leaves (183.57 ± 1.18 µg/g dw in petioles and 86.00 ± 0.74 µg/g dw in leaf blades). In petioles, the most abundant biflavonoid was bilobetin (984.48 ± 6.42 µg/g dw), followed by isoginkgetin (883.83 ± 5.41 µg/g dw), sciadopitysin (727.14 ± 2.98 µg/g dw), and ginkgetin (627.22 ± 3.21 µg/g dw). In leaf blades, the most abundant biflavonoid was sciadopitysin (2398.59 ± 6.11 µg/g dw), followed by isoginkgetin (1896.02 ± 11.92 µg/g dw) and bilobetin and ginkgetin, whose contents were similar and were 1378.34 ± 11.22 µg/g dw and 1331.17 ± 5.85 µg/g dw, respectively.

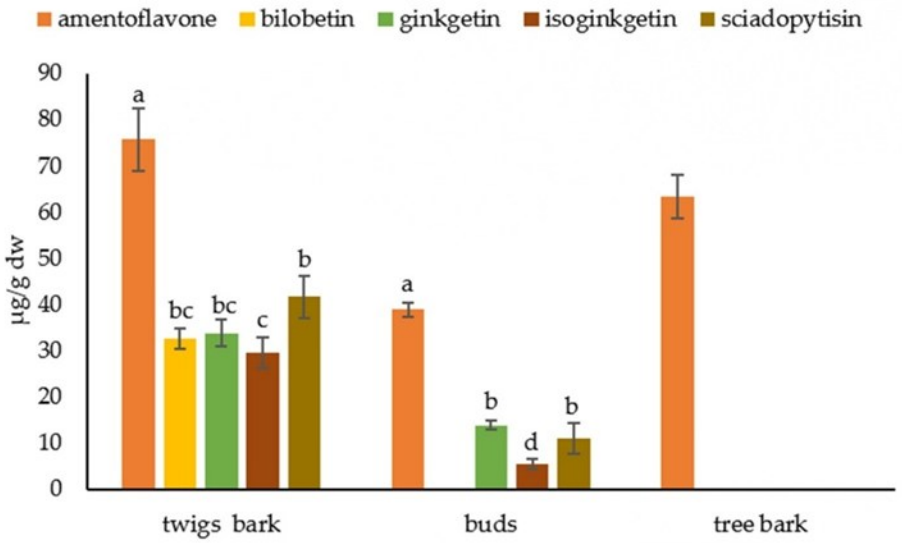


Figure 5. Biflavonoid profile in tree and twig bark and buds. Biflavonoid levels labeled with different letters differ significantly at $p < 0.05$ within the plant part.

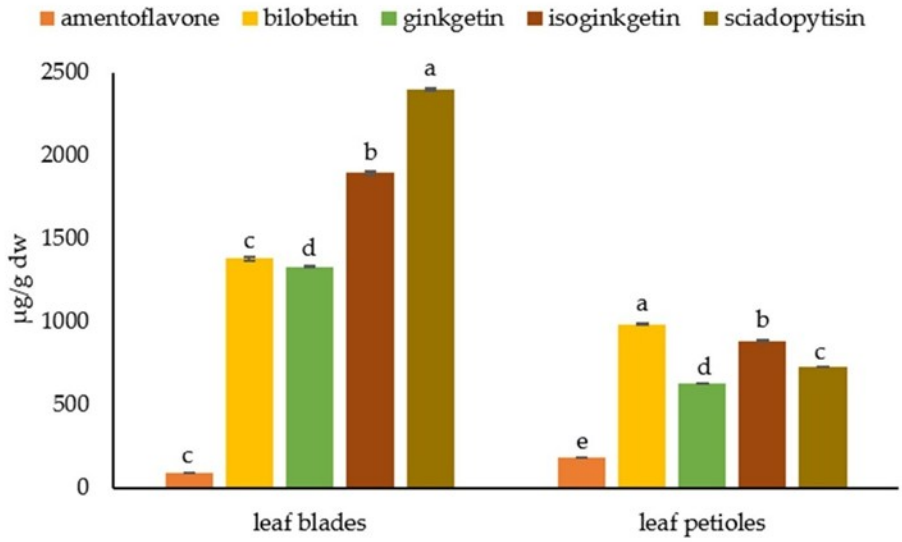


Figure 6. Biflavonoid profile in leaf parts- leaf blade and petiole. Biflavonoid levels labeled with different letters differ significantly at $p < 0.05$ within the plant part.

We analyzed seed petioles, sarcotesta, nutshells, and kernels separately and detected biflavonoids only in seed petioles and sarcotesta (Figure 7). In seed petioles, the most abundant biflavonoid was isoginkgetin (380.41 ± 26.98 µg/g dw), while the least abundant biflavonoid was amentoflavone (34.01 ± 2.51 µg/g dw). Other biflavonoids were present in the following concentrations: ginkgetin 146.62 ± 9.37 µg/g dw, bilobetin 249.29 ± 19.23 µg/g dw and sciadopitysin 289.32 ± 19.19 µg/g dw. In a sarcotesta, the biflavonoids were present in similar proportions. The largest abundant was isoginkgetin (311.67 ± 16.92 µg/g), followed by sciadopitysin (224.32 ± 4.44 µg/g dw), bilobetin (138.34 ± 49.19 µg/g dw), and ginkgetin (116.53 ± 6.58 µg/g dw). The least abundant biflavonoid in sarcotesta was amentoflavone with a concentration of 17.52 ± 2.77 µg/g dw.

The total content of biflavonoids in different parts of the ginkgo plant is shown in Figure 8. As you can see, the biflavonoid content is highest in the leaves, followed by the seeds, while the other parts have much lower biflavonoid content.

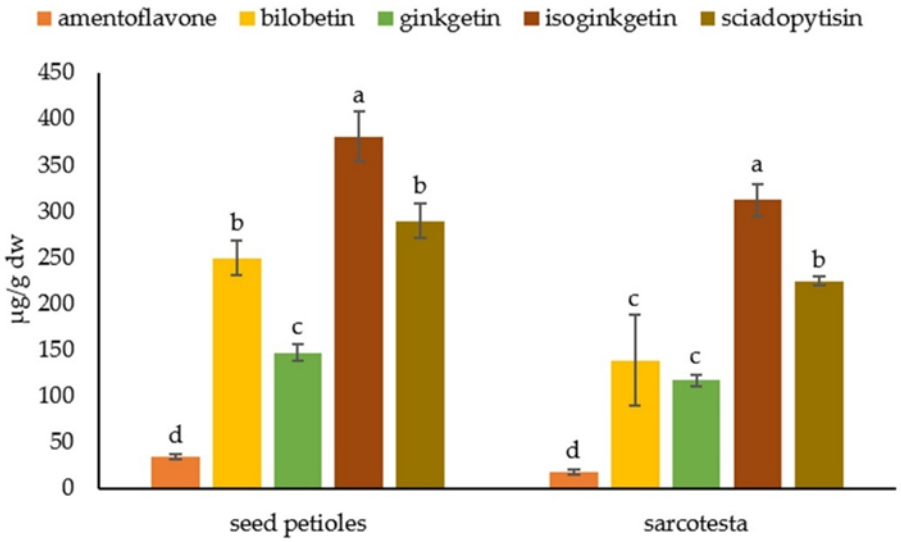


Figure 7. Biflavonoid profile of the seed parts. Biflavonoid levels labeled with different letters differ significantly at $p < 0.05$ within the plant part.

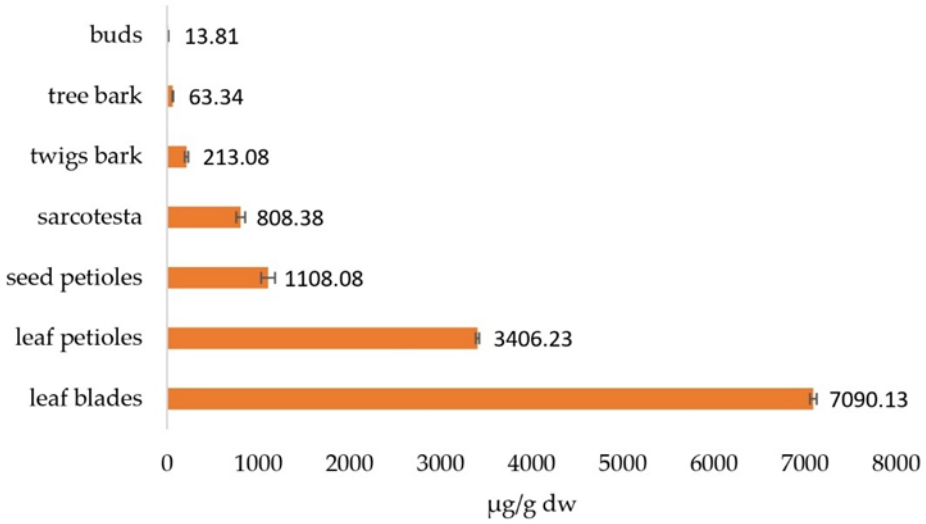


Figure 8. Total biflavonoid content in analyzed ginkgo parts.

3. Discussion

In the past, separation and especially identification of biflavonoids was a difficult task due to lack of modern analytical equipment and commercially available standards. Many of the early reported methyl biflavones had to be corrected for structure, as structural elucidation in the 1960s and 1970s depended on co-chromatography with isolated authentic compounds, which in turn could be misidentified [18]. Biflavonoids have a strong signal at 330 nm, making the DAD detector in combination with standards a compelling and rapid method for detecting biflavonoids in ginkgo. One of the earliest HPLC-DAD methods for separating biflavonoids from ginkgo leaves was developed by Briancon-Scheid et al. [19], the HPLC-DAD method for the simultaneous determination of four (bilobetin, ginkgetin, isoginkgetin, sciadopitysin) biflavonoids. Kaur et al. [20] developed a method for the simultaneous determination of three biflavonoids (bilobetin, sciadopitysin, and ginkgetin) from ginkgo leaves, and recently Lei et al. [21] focused on four biflavonoids (bilobetin, ginkgetin, isoginkgetin, sciadopitysin), as did Wang et al. [22]. Pandey et al. [23] reported only two biflavonoids (amentoflavone and sciadopitysin) in a crude extract of ginkgo leaves. Beck and Stengel [7] like us, reported five biflavonoids in ginkgo leaves but were unable to separate ginkgetin from isoginkgetin. To determine the content of biflavonoids in

different plant parts (tissues) of ginkgo, we have developed the HPLC-DAD method for the separation and simultaneous quantification of the five major biflavonoids in ginkgo [3], which allows us to profile different tissue types.

We analyzed ten plant parts and could not detect any of the analyzed biflavonoids in three parts (twigs without bark, nutshells, and seeds). Chen et al. [24] also failed to detect biflavonoids in seeds or embryoids, as they called this the seed part. We detected biflavonoids in all parts studied that are in contact with the environment, which may indicate a biological role for biflavonoids in the context of plant-environment interactions. This is consistent with literature data that biflavonoids are localized in the outer part of the leaves of *G. biloba* [7] and above the ground rhizome of *Psilotum nudum* [8] according to MALDI imaging data.

We found the highest total biflavonoid content and the presence of all five biflavonoids in the leaf blades, followed by the petioles. In the leaf blades, the most abundant biflavonoid was sciadopitysin, which is consistent with the report of Wang et al. [22] who also described sciadopitysin as the most abundant biflavonoid in yellow ginkgo leaves. In leaf parts, we detected a much lower amount of amentoflavone compared with other biflavonoids, suggesting that more methylated biflavonoids are present in leaves. A similar high amount of sciadopitysin compared to amentoflavone in leaves was reported by Pandey et al. [23] in ginkgo trees of different ages. The low content of amentoflavone is probably the reason why this biflavonoid was generally not detected in leaves in other studies [19,21,22]. Amentoflavone was also the least abundant biflavonoid in the seed parts where biflavonoids were detected in seed petioles and sarcotesta, according to our data. Chen et al. [24] reported only isoginkgetin in ginkgo sarcotesta at concentrations ranging from 179.78 to 424.42 μg/g dry weight, which is comparable to our results where isoginkgetin was the most abundant biflavonoid (380.41 ± 26.98 μg/g dw). Shen et al. [25] reported bilobetin, isoginkgetin, and ginkgetin in sarcotesta and developed a method for large-scale targeted isolation of these biflavonoids from the exocarp (sarcotesta), which is an industrial waste.

In contrast to the leaves and seeds, in which methylated biflavonoids dominate, the most abundant biflavonoid in the bark and buds of trees and twigs was amentoflavone. In the bark, we could detect only amentoflavone and at the same time, other biflavonoids were below the detection limit. In the bark of the twig, we also detected other biflavonoids, but the amentoflavone content was twice as high as in other parts. Pandey et al. [23] reported the presence of amentoflavone and sciadopitysin in ginkgo stems, but the authors did not specify which type of stems they analyzed. The presence of amentoflavone has also been reported in the bark of other plants such as *Calophyllum pinetorum* [26], *Ochna schweinfurthiana* [27] and *Anacolosa poilanei* [28]. If we consider the strong antimicrobial [29,30] and antiparasitic [31–33] activity of amentoflavone, this could indicate its role in protection against biotic stress, but further studies should test this hypothesis.

4. Materials and Methods

4.1. Materials and Plant Samples

Ultrapure water was prepared using the Purelab flex system (ELGA LabWater, Wycombe, UK) and all solvents used were of HPLC grade. Certified analytical standards of biflavonoids, including amentoflavone (CAS 1617-53-4), bilobetin (CAS 521-32-4), ginkgetin (CAS 481-46-9), isoginkgetin (CAS 548-19-6), and sciadopitysin (CAS 521-34-6), were purchased from Phytolab (Vestenbergsgreuth, Germany). Plants of *Ginkgo biloba* L. were collected in Koprivnica, Croatia, in late October 2022. Leaves, seeds and bark samples were collected and then separated to obtain different tissues (Figure 4). Fresh material was quickly frozen and freeze-dried using a laboratory freeze dryer (LIO-5PLT, KAMBIČ, Ljubljana, Slovenia). The dry plant material was pulverized using a bead mill (Bead Ruptor 12, Omni International, USA), sealed under vacuum and stored at room temperature for further experiments.

4.2. Preparation of Standard Solutions

Standard stock solutions of five biflavonoids (amentoflavone, bilobetin, sciadopitysin, ginkgetin and isoginkgetin) were prepared individually in DMSO in a concentration of 1000 µg/mL. An appropriate amount of each standard stock solution was diluted with 80% methanol to obtain seven working standard solutions (concentrations 1.0, 2.0, 5.0, 10, 20, 50 and 100 µg/mL) for constructing the relevant calibration curves. The standard stock solution, along with the working solutions, was kept in dark at −20 °C until use.

4.3. Biflavonoids Extraction

Powdered samples of various *G. biloba* L. plant parts were accurately weighed (30 mg) and sonicated for 10 min at room temperature in the presence of extraction solvent (1 mL of 80% methanol), followed by a 45 min rotation in a mechanical shaker (Bio RS-24, Biosan, Latvia) and 5 min centrifugation (LMC-4200R, Biosan, Latvia) at 4000× *g*. Prior to the instrumental analysis, the obtained sample extracts were filtered through a 45 µm pore size polytetrafluoroethylene syringe filter. Each plant sample type was prepared in triplicates after which the average result value was expressed for each biflavonoid compound.

4.4. HPLC-DAD Analysis

The instrumental analysis was performed using an Agilent 1260 Infinity II HPLC system (Agilent, Santa Clara, CA, USA), equipped with a quaternary pump system, autosampler, column department and diode array detector (DAD). The acquisition and data processing was conducted using Agilent OpenLAB CDS software (v. 2.6, Agilent, Santa Clara, CA, USA). Chromatographic separation was attained using Zorbax 300Extend-C18 column, 150 × 4.6, 3.5 µm (Agilent, Santa Clara, CA, USA) maintained at 30 °C, and 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) at a constant flow rate of 1.0 mL/min during 42 min of the analysis. The used multistep linear solvent gradient for the analyte's elution was as follows: 0 min 98% A, 5 min 90% A, 15 min 70% A, 20 min 50% A, 25 min 50% A, 30 min 20% A, 32 min 2% A, 40 min 98% A. The injection volume was 10 µL for both standards and samples. UV/Vis spectra were recorded in the range of 190–400 nm, and the chromatograms were acquired at 330 nm.

4.5. Statistical Analysis

For analysis, we collected samples from six different plants, three of which were female from which we took seeds. We all collected material pulled together and for analysis, we performed three separate extractions and analyzed them on HPLC-DAD. Statistical analyses were performed using the free software PAST [34]. One-way ANOVA and post hoc multiple means comparison (Tukey's HSD test) were performed and differences between measurements were considered significant at $p < 0.05$.

5. Conclusions

In the present study, we developed a method for the simultaneous determination of five biflavonoids (amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin) and performed tissue-specific profiling in ten different parts of ginkgo plants. We did not detect biflavonoids in three plant parts (twigs without bark, nutshells, and kernels) that were not in direct contact with the environment. In the other plant parts studied, biflavonoids were most abundant in leaves, followed by seeds. In bark samples and buds, the predominant biflavonoid was amentoflavone, which, in contrast, was least abundant in leaves and seeds, where methylated biflavonoids dominated. In petioles and leaf blades, the most abundant biflavonoid was sciadopitysin, and in petioles and the sarcotesta of seeds, isoginkgetin. The accumulation of biflavonoids in the parts in direct contact with the environment probably indicates their role in plant-environment interaction. However, the different accumulation of each biflavonoid might be related to their chemical structure and different biological functions. Further studies should focus on elucidating the role of biflavonoids in plants, with emphasis on the structural functional differences.

Author Contributions: Conceptualization, D.Š. and M.K.T.; methodology, M.K.T., D.Š. and I.J.; validation, M.K.T.; investigation, M.K.T., D.Š. and I.J.; formal analysis, M.K.T.; resources, I.J. and D.Š.; writing—original draft preparation, D.Š. and M.K.T.; writing—review and editing, D.Š., M.K.T. and I.J.; visualization, D.Š.; supervision, D.Š.; project administration, D.Š.; funding acquisition, D.Š. All authors have read and agreed to the published version of the manuscript.

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5.1.3. Seasonal Variation of Polyphenols and Pigments in Ginkgo (*Ginkgo biloba* L.) Leaves: Focus on 3',8''-Biflavones

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Seasonal Variation of Polyphenols and Pigments in Ginkgo (*Ginkgo biloba* L.) Leaves: Focus on 3′,8″-Biflavones

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Abstract: Ginkgo (*Ginkgo biloba* L.) is a widely recognized medicinal plant, often grown as an ornamental species in parks around the world. Its leaves change color from green in spring to yellow in autumn. In this study, we collected ginkgo leaves at seven developmental stages from May to November and measured chlorophylls, carotenoids, flavonoids, and antioxidant activity. The total polyphenol content showed a significant increase from May to November, rising from 15.15 ± 0.14 mg GAE g^{-1} dw to 45.18 ± 0.42 mg GAE g^{-1} dw. The total flavonoid content reached its peak in August at 5.87 ± 0.18 mg GAE g^{-1} dw. In contrast, the highest concentrations of total polyphenolic acids (4.13 ± 0.16 mg CAE g^{-1} dw) and antioxidant activity (306.95 ± 3.47 µmol TE g^{-1}) were recorded in May. We specifically focused on a less-studied group of dimeric flavonoids or biflavonoids—3′,8″-biflavones. We identified five 3′,8″-biflavones (amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin) throughout all developmental stages. Sciadopitysin was the most abundant biflavonoid, with its concentration rising from 614.71 ± 5.49 µg g^{-1} dw in May to 2642.82 ± 47.47 µg g^{-1} dw in November. Alongside sciadopitysin, the content of other biflavonoids (excluding amentoflavone) generally increased over the same period. This trend is further highlighted by the total biflavonoid content, which grew from 1448.97 ± 6.63 µg g^{-1} dw in May to 6071.67 ± 97.15 µg g^{-1} dw in November. We observed a negative correlation between biflavonoid and chlorophyll content, which may indicate their involvement in leaf senescence. However, this hypothesis warrants further investigation.

Keywords: biflavonoids; *Ginkgo biloba* L.; leaves; phenolic compounds

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1. Introduction

Flavonoids are diverse group of specialized plant metabolites. Their chemical structure is based on a C6-C3-C6 skeleton, consisting of two benzene rings (rings A and B) connected by a 3-carbon chain, which often forms a third ring (ring C). Due to structural variations, flavonoids are classified into several categories, including flavanones, flavanols, flavones, and flavonols. Other classes of flavonoid compounds include isoflavones, biflavonoids, flavonolignans, prenylflavonoids, flavonoid glycoside esters, aurones, and chalcones [1]. Structural modifications can vary and include processes such as hydroxylation, methylation, and glycosylation. All these structural features of flavonoids may influence their biological activities [2]. Flavonoids, despite being known as secondary metabolites of plants, play a significant role in various plant functions, particularly in the context of plant–environment interactions and a more appropriate name for them would be specialized metabolites. In plants, they serve as defences against pathogenic insects and fungi, act as antioxidants, and function as antimicrobial agents [3]. Flavonoids are widely recognized as molecules with significant biological activity and health-promoting properties [1].

Ginkgo (*Ginkgo biloba* L.) is a medicinal plant, known also as a living fossil and the only surviving member of the Ginkgoaceae family. The unique botanical features of ginkgo are most evident in its fan-shaped leaves with dichotomous venation, which, along with

their seasonal colour changes, contribute to the ornamental value of this species. Although this species originates from China, it is now widespread across the globe. Ginkgo is characterised by its remarkable resilience to various abiotic and biotic stresses. A testament to this resilience is the fact that several specimens survived the nuclear bombing of Hiroshima [4]. Specialized metabolites are considered one of the key factors contributing to ginkgo's excellent adaptation to various environmental conditions [5]. Ginkgo has long been recognised in traditional medicine as a source of phytochemicals beneficial for health and is often mentioned in the context of cardiovascular diseases, cancer, cognitive dysfunctions, asthma, bronchitis, and more [4]. The health benefits of ginkgo are attributed to its rich composition of bioactive components such as flavonoids, terpenes, and trilactones [6]. The European Pharmacopoeia proposed that the standard leaf extract contains 5–7% terpene lactones and 22–27% flavone glycosides [7]. According to the review by Liu et al. [6], 110 flavonoids have been detected in ginkgo, most commonly in the form of aglycones, glycosides, and biflavonoids.

Biflavonoids, dimeric forms of flavonoids, consist of either two identical or different flavonoid units and most commonly form combinations such as flavone–flavone, flavone–flavanone, and flavanone–flavanone. Biflavonoids are particularly associated with plants used in both traditional and modern medicine [3]. Dimerization influences their biological activity [8], positioning them as a key focus for research and the potential development of new pharmaceuticals. According to He et al. [9], approximately 600 different biflavonoids have been reported, which are divided into 24 subgroups based on the position of the C-C linkage. To date, 13 biflavonoids have been identified in ginkgo, with the most common being amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin [3]. The biflavonoids found in ginkgo belong to the flavone group, which forms a bond at the 3′C–8″C position and are therefore known as 3′,8″-biflavones. They are recognized as compounds with potential pharmacological applications in the treatment of various cancers, neurological and cardiovascular disorders, and as antiviral agents [3,10–12]. The highest concentrations of 3′,8″-biflavones have been recorded in the leaves of ginkgo, as well as in other parts of the plant directly exposed to the environment, such as bark, petioles, and seed sarcotesta [13]. Given their localisation, they may play a role in protecting against pathogens and mitigating the effects of UV radiation, but their exact role in plants remain unknown [13].

Flavonoids serve as biomarkers of the external environment, with factors such as light, drought, temperature, and nutrient availability playing a crucial role in their synthesis and accumulation in ginkgo leaves [14]. Consequently, the timing of leaf harvesting naturally exerts a significant influence on the composition of phytochemicals [15–20]. Previous research on ginkgo has primarily focused on variations in flavonoid glycosides, including quercetin, kaempferol, and isorhamnetin, with the goal of identifying differences in their content between green and yellow leaves [19,21]. Kobus-Cisowska et al. [21] associate the increased content of flavonoid compounds with the higher antioxidant activity of autumn extracts. Similar results were published by Zheng et al. [19], who compared methanol extracts of ginkgo leaves collected in June and October. Comparative metabolomic and transcriptomic studies suggest a potential role for flavonoid compounds in the regulation of leaf senescence [22,23].

However, data on biflavonoids, particularly 3′,8″-biflavones, are limited, with no studies to date reporting their seasonal variation. Therefore, this study aims to investigate the seasonal variation of individual biflavonoids in ginkgo leaves. We profiled the distribution of the five most prevalent 3′,8″-biflavones—amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin—in ginkgo leaves throughout the entire growing season, from May to November (seven development stages). We also correlated their content with pigments, total polyphenols, flavonoids, phenolic acids, and antioxidant activity (using the DPPH radical assay).

2. Results and Discussion

2.1. Seasonal Variation of Pigments in Ginkgo Leaves

Throughout the growing season, we monitored the change in leaf color. As part of a normal physiological process, leaves change color from green in May to yellow in October, as shown in Figure 1.

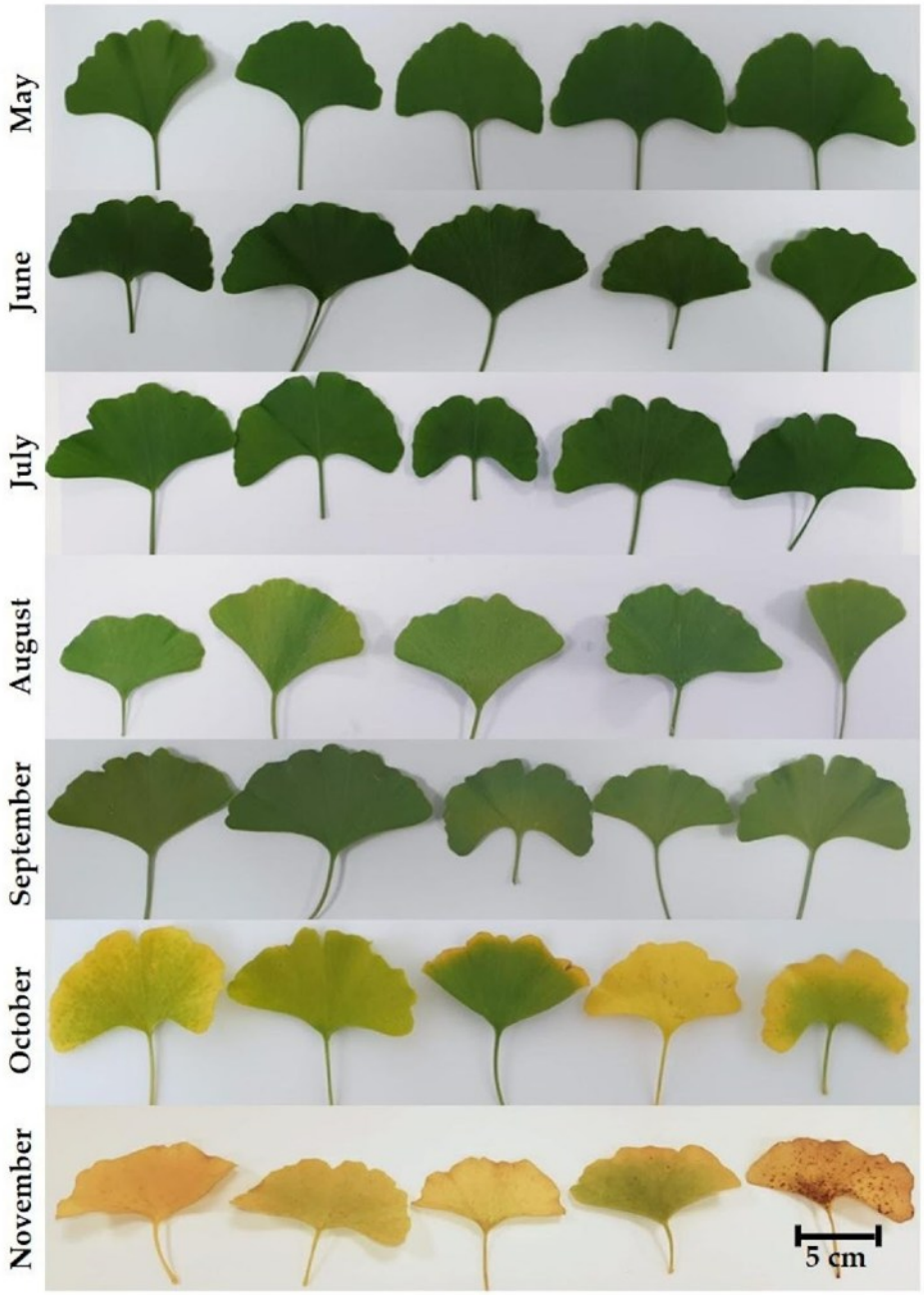

Figure 1. Ginkgo leaves during the 2022 growing season from May, June, July, August, September, October, and November (from top to bottom).

Changes in leaf color are linked to variations in pigment content, so we measured the levels of photosynthetic pigments. The levels of chlorophyll *a* and *b*, as well as total chlorophylls, total carotenoids, and the ratios of chlorophyll a to b and chlorophylls to carotenoids throughout the growing season of ginkgo leaves are shown in Table 1.

Table 1. Monthly variation of pigments (chlorophyll *a*, chlorophyll *b*, total chlorophylls, total carotenoids, and the ratios of chlorophyll *a* to *b* and chlorophylls to carotenoids) in ginkgo leaves throughout the growing season. Values marked with different letters are significantly different at $p < 0.05$.

Season 2020	Chlorophyll *a* (mg g $^{-1}$ dw)	Chlorophyll *b* (mg g^{-1} dw)	Total Chlorophylls (mg g^{-1} dw)	Total Carotenoids (mg g^{-1} dw)	Chlorophyll *a*/Chlorophyll *b*	Chlorophylls/ Carotenoids
May	0.68 ± 0.04 ^{c}	0.15 ± 0.04 ^{bc}	0.84 ± 0.08 ^{c}	0.02 ± 0.00 ^{e}	4.73 ± 0.91 ^{a}	56.65 ± 18.03 ^{a}
June	0.96 ± 0.04 ^{b}	0.18 ± 0.02 ^{b}	1.13 ± 0.06 ^{b}	0.03 ± 0.00 ^{de}	5.42 ± 0.29 ^{a}	34.09 ± 0.65 ^{ab}
July	1.71 ± 0.06 ^{a}	0.35 ± 0.01 ^{a}	2.07 ± 0.07 ^{a}	0.24 ± 0.01 ^{a}	4.83 ± 0.09 ^{a}	8.57 ± 0.06 ^{c}
August	0.87 ± 0.01 ^{b}	0.17 ± 0.01 ^{b}	1.04 ± 0.02 ^{b}	0.05 ± 0.01 ^{d}	5.08 ± 0.21 ^{a}	20.35 ± 2.12 ^{bc}
September	0.73 ± 0.01 ^{c}	0.13 ± 0.0 ^{bcd}	0.86 ± 0.01 ^{c}	0.05 ± 0.00 ^{d}	5.62 ± 0.17 ^{a}	17.26 ± 0.61 ^{bc}
October	0.20 ± 0.01 ^{d}	0.09 ± 0.01 ^{cd}	0.29 ± 0.02 ^{d}	0.08 ± 0.00 ^{c}	2.14 ± 0.21 ^{b}	3.69 ± 0.39 ^{c}
November	0.07 ± 0.00 ^{e}	0.08 ± 0.01 ^{d}	0.15 ± 0.01 ^{d}	0.11 ± 0.01 ^{b}	0.80 ± 0.08 ^{c}	1.32 ± 0.16 ^{c}

The levels of chlorophyll *a*, chlorophyll *b*, and total chlorophyll in ginkgo leaves follow a similar trend throughout the growing season. Both types of chlorophyll steadily increase from May, peaking in July with concentrations of 1.71 ± 0.06 μg g^{-1} dw for chlorophyll *a* and 0.35 ± 0.01 μg g^{-1} dw for chlorophyll *b*. After reaching these maximum levels, the chlorophyll concentrations gradually decrease until the end of the growing season. The ratio of chlorophyll *a* to *b* remains stable throughout most of the growing season, but shows a significant decrease in the autumn leaf samples from October and November. A decrease in the chlorophyll *a*/*b* ratio occurs during leaf senescence. During this process, the chlorophyll *a* degrades more rapidly than chlorophyll *b*, leading to a relative increase in the proportion of chlorophyll *b*. This change can be associated with the enlargement or restructuring of the antenna system of Photosystem II (PS II) as the plant prepares for the end of the growing season [24]. The trend of decreasing chlorophyll content during senescence has already been observed in ginkgo trees. Zhang et al. [25] and Li et al. [22] reported that chlorophyll levels in ginkgo leaves begin to decline in early September, signalling the onset of autumn. This observation is consistent with our results.

The total carotenoid content, wh exhibited greater variation throughout the growing season. It increased from May to July, peaking during this period. This was followed by a significant decline in concentration from August to September, after which the levels rose again towards the end of the growing season. This trend is expected, as the yellow color of autumn leaves is traditionally associated with carotenoids. As chlorophyll degrades, the green pigment in the leaves diminishes, leading to the gradual accumulation of significant amounts of carotenoids and a color change [26]. The significant drop in chlorophyll observed in October may accelerate leaf senescence. However, since carotenoids did not dominate over chlorophyll, the yellowing of ginkgo leaves cannot be attributed to carotenoids alone. Other pigments, such as flavonoids, may also contribute to the yellow coloration. The mechanism underlying leaf coloration in ginkgo remains poorly understood and it may be that flavonoids are associated with the yellow coloration in addition to carotenoids [23].

2.2. Seasonal Variation of Total Polyphenols, Total Flavonoids, and Total Phenolic Acids in Ginkgo Leaves

Polyphenolic compounds may also contribute to the leaf's coloration [27]. Thus, we determinate the total polyphenols, flavonoids, and phenolic acids content during the growing season (Figure 2).

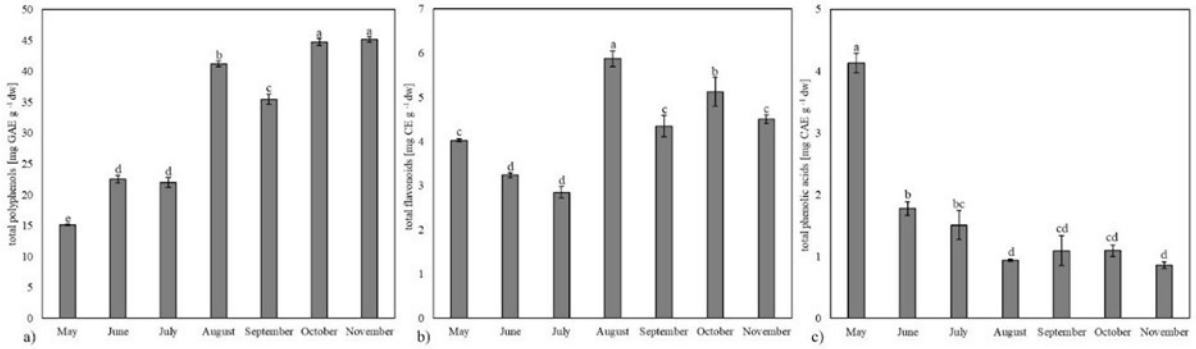

Figure 2. Total polyphenols (**a**), flavonoids (**b**), and phenolic acids (**c**) in ginkgo leaves during the growing season. Values marked with different letters are significantly different at $p < 0.05$.

The total polyphenol content increases from the beginning of the season until August, after which there is a decline in concentration. The highest value of polyphenols was recorded in leaf samples collected at the end of the growing season, in October (44.75 ± 0.55 mg GAE g^{-1} dw) and November (45.18 ± 0.42 mg GAE g^{-1} dw). In the case of flavonoids, the highest content was observed in the leaves collected in August (5.87 ± 0.18 mg CE g^{-1} dw), followed by a notable presence in the autumn leaves from October, as well as in September and November. These results are consistent with studies suggesting that increased flavonoid synthesis in ginkgo is associated with higher levels of UV-B radiation [5,28,29]. In ginkgo, the expression pattern of genes related to flavonoid biosynthesis is mainly driven by environment [5]. Zhao et al. [30] exposed ginkgo seedlings to UV-B radiation, and observed increased expression of FLS and F3′H and increased content of quercetin, kaempferol, and isorhamnetin. Xu et al. [29] analyzed flavonols (total flavonol, quercetin, kaempferol, and isorhamnetin) content, flavonol yield per plant, and expression of flavonoid biosynthesis-related genes in 2-year ginkgo at four different light intensities (100, 76, 40, and 25% of full sunlight). The 100% sunlight produced the highest flavonoid content, which is with line with our results, which show the highest content of flavonoids in samples collected during the month when the sunlight is the strongest. Consistent with our findings, Rimkiene et al. [17] recommended July and August as the optimal months for harvesting ginkgo leaves to maximize the yield of flavonoids such as quercetin, kaempferol, and isorhamnetin. According to the review article by Mao et al. [31], the role of flavonoids in ginkgo is closely related to environmental conditions, particularly in protecting the plant from UV-B radiation, drought stress, and biotic stress, including antiviral and antifungal activities. Their synthesis is regulated by several transcription factors, including MYB, bZIP, bHLH, WD40, NAC, and TCP. Additionally, hormones, miRNAs, and lncRNAs also play a role in regulating flavonoid synthesis. The review notes that the flavonoid content in the leaves of young trees is significantly higher than that in adult trees, indicating that only the leaves of young trees are suitable as raw materials for drug production, thereby highlighting the importance of tree age in flavonoid accumulation. However, our findings, which align with other studies [17,28,29], suggest that certain flavonoids may also increase during later developmental stages. According to our results, only phenolic acids were significantly higher in May (4.13 ± 0.16 CAE mg^{-1} dw), followed by a decline in their content in the subsequent months. These results are similar to studies on free and bound phenolic acids in leaves of *Centaurea* sp. [32] and walnuts (*Juglans regia* L.) [33], where the highest content of phenolic acids was recorded at the beginning of the growing season.

2.3. Seasonal Variation of 3′–8″ Biflavones in Ginkgo Leaves

As mentioned above, the color of yellow leaves may be due to the presence of flavonoids, which have a yellow color. In 1929, the yellow pigment was isolated from yellow gingko leaves [34], and identified as ginkgetin, a dimeric flavonoid which belongs

to the group of 3′,8″-biflavones [35]. Later, isoginkgetin, biolobetin, sciadopitysin and amentoflavone were also reported in ginkgo leaves as a main 3′,8″-biflavones [13,36–38]. The content of those five main biflavones as well the total biflavones content during the growing season in ginkgo leaves are shown on Figure 3.

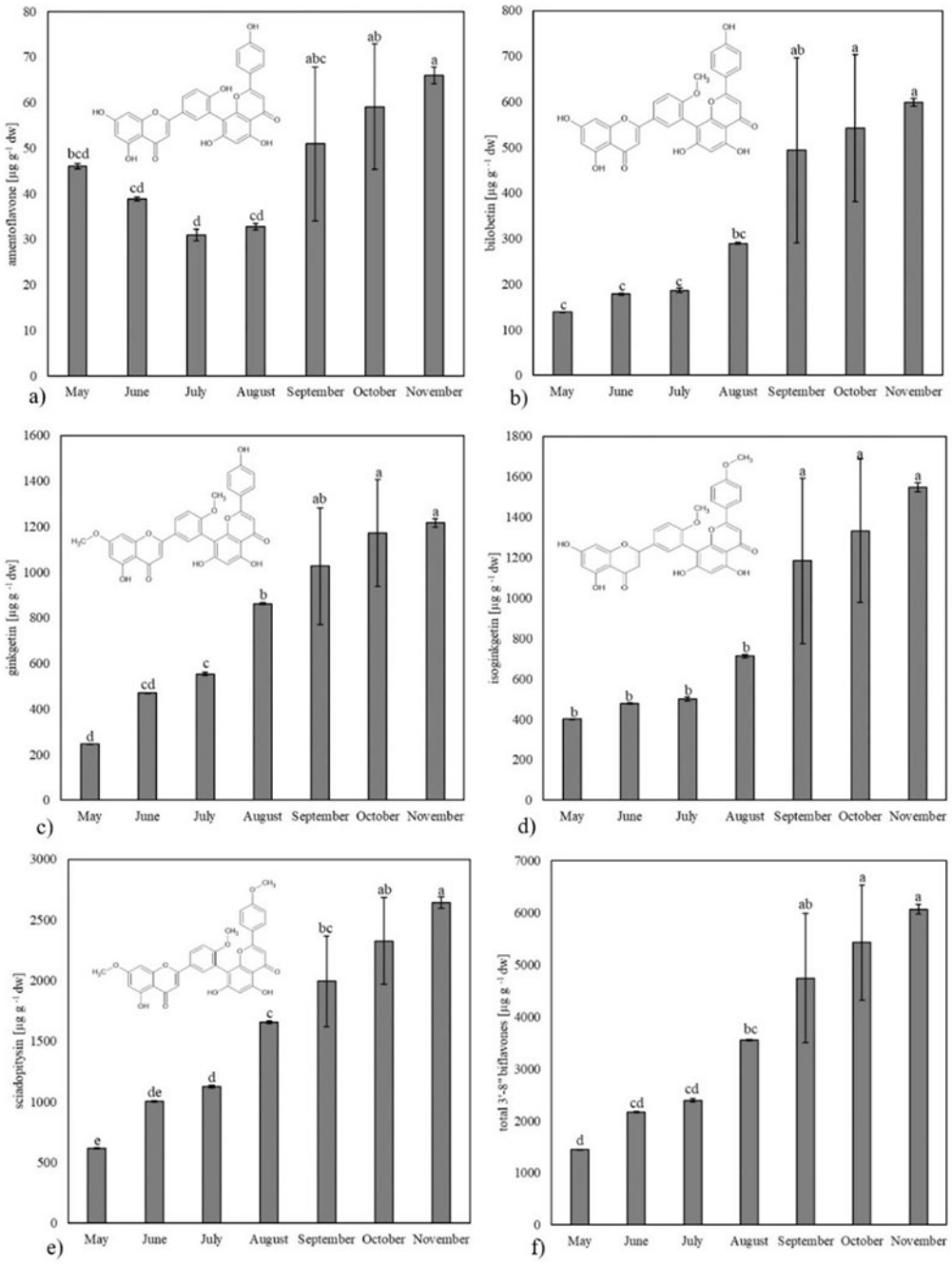

Figure 3. The content of biflavonoids during the season: amentoflavone (**a**), bilobetin (**b**), ginkgetin (**c**), isoginkgetin (**d**), sciadopitysin (**e**), and total 3′,8″-biflavones (**f**) in ginkgo leaves during the growing season. Values marked with different letters are significantly different at $p < 0.05$.

Throughout the growing season, the ratio of identified biflavones remains stable, with sciadopitysin being the most abundant, ranging in concentration from 614.74 to 2642.82 µg g^{-1} dw. This aligns with reports in the literature, which identify sciadopitysin as the most abundant biflavone in ginkgo leaves [13,38–40]. Biflavones named after ginkgo, such as isoginkgetin and ginkgetin, were detected in significantly lower amounts

than sciadopitysin. Ginkgetin levels ranged from 247.61 to 1215.98 μg g^{-1} dw, while isoginkgetin concentrations varied between 401.35 and 1547.95 μg g^{-1} dw. Amentoflavone was the least prevalent among the biflavonoids, with concentrations ranging from 30.87 to 65.96 μg g^{-1} dw, followed by bilobetin, which ranged from 139.23 to 598.96 μg g^{-1} dw. According to the literature, geolocation [16] and the age [41] of the tree may influence biflavonoid composition. However, our data indicate that the ratio of biflavonoids remains consistent throughout the growing season. Notably, we observed an increasing trend in the total biflavonoid content from May to November for all biflavonoids except amentoflavone, which followed a distinct pattern. Studies on the seasonal variation of biflavonoids in ginkgo and other plants are quite limited. Yang et al. [42] examined the seasonal variation of the biflavonoids ginkgetin and amentoflavone in the needles of Chinese yew (*Taxus wallichiana* var. *maire*). The highest concentration of ginkgetin was observed in needles collected in August, while amentoflavone levels fluctuated throughout the season without a clear increasing or decreasing trend. The authors found no correlation between flavonoid accumulation and environmental factors such as temperature, daylight length, relative humidity, or rainfall. Wang et al. [39] reported the levels of bilobetin, ginkgetin, isoginkgetin, and sciadopitysin at six developmental stages of yellowing ginkgo leaves collected from mid-October to mid-November. Only sciadopitysin showed higher concentrations at the later stages, while the other biflavonoids exhibited significant fluctuations, with some not being detected just in some developmental stages. The study lacked precise details on leaf collection protocols and biological replicates, which may have affected the reliability of the results. Our data show that, in the later developmental stages, there is a larger standard deviation and greater variability in results between trees compared to younger leaves. Accurate leaf sampling is therefore critical for tracking the dynamics of biflavonoid accumulation effectively.

2.4. Seasonal Variation of Antioxidant Activity in Ginkgo Leaves

As we have previously shown, the composition of phytochemicals in ginkgo leaf extract varies throughout the season. Consequently, the ability to scavenge free radicals also changes during different growth periods. Antioxidant activity in ginkgo leaves throughout the growing season is illustrated in Figure 4.

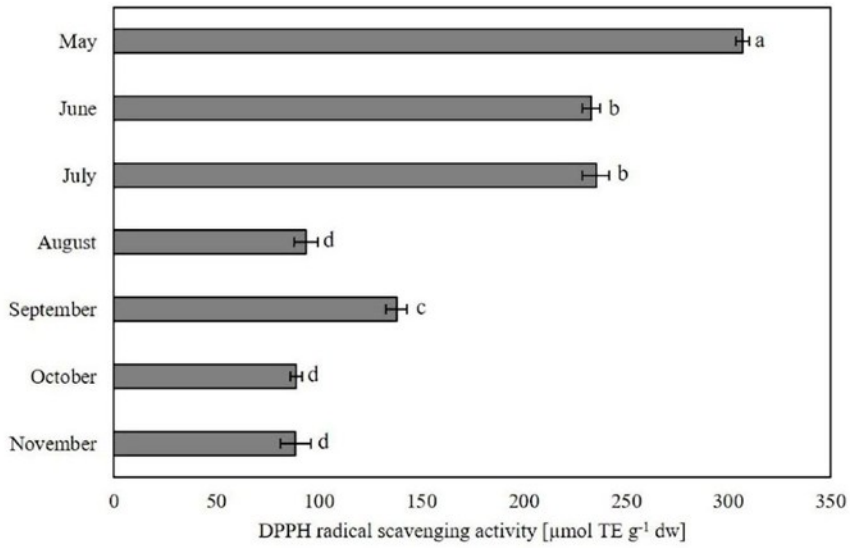

Figure 4. Antioxidant activity measured by DPPH in ginkgo extracts prepared from leaves collected during the growing season. Values marked with different letters are significantly different at $p < 0.05$.

The highest antioxidant activity was recorded in leaves harvested at the beginning of the season in May (306.95 ± 3.47 μmol TE g^{-1} dw), followed by a gradual decline until August (93.75 ± 5.85 μmol TE g^{-1} dw). A significant increase in antioxidant activity was

observed in the September samples (137.91 ± 4.96 µmol TE g^{-1} dw), while the levels in leaves collected at the end of the growing season were comparable to those measured in August. These results contrast with the findings of Kobus-Cisowska et al. [21], who reported higher antioxidant activity in ginkgo leaf extracts from yellow, autumn leaves in October compared to summer leaves from August, as determined by DPPH free radical assays. The difference was attributed to the increased content of flavonoids such as quercetin, rutin, myricetin, morin, isorhamnetin, and kaempferol, as well as total phytosterols. Our observed trend in antioxidant activity correlates with the dynamics of phenolic acids, which are notably higher in the first part of the growing season. Although levels of other phenolic compounds, including 3′,8″-biflavones, increase from August onwards, these biflavones have not demonstrated significant antioxidant activity in *in vitro* systems [8].

2.5. Correlation Analysis of Measured Parameters

To determine the correlations between the measured parameters, we created a correlation matrix, as shown in Table 2.

Table 2. Correlation matrix of measured parameters, in ginkgo leaves during season.

	chl a	chl b	Total chls	Total car	DPPH	TP	TF	TPA	a	b	g	i	s	TB
chl *a*	1													
chl *b*	0.961	1												
total chls	0.999	0.971	1											
total car	0.504	0.696	0.53	1										
DPPH	0.551	0.508	0.547	−0.016	1									
TP	−0.637	−0.576	−0.631	−0.007	−0.992	1								
TF	−0.592	−0.619	−0.598	−0.432	−0.730	0.744	1							
TPA	0.133	0.100	0.129	−0.334	0.851	−0.796	−0.330	1						
a	−0.899	−0.808	−0.890	−0.207	−0.464	0.556	0.289	−0.163	1					
b	−0.743	−0.669	−0.736	0.006	−0.840	0.877	0.496	−0.647	0.829	1				
g	−0.622	−0.551	−0.614	0.093	−0.948	0.960	0.581	−0.807	0.658	0.961	1			
i	−0.759	−0.671	−0.750	0.024	−0.822	0.865	0.470	−0.625	0.853	0.997	0.950	1		
s	−0.674	−0.584	−0.664	0.100	−0.921	0.945	0.541	−0.767	0.726	0.975	0.991	0.973	1	
TB	−0.702	−0.617	−0.693	0.067	−0.898	0.927	0.530	−0.728	0.766	0.990	0.987	0.987	0.997	1

Abbreviations: chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*), total chlorophylls (total chls), total carotenoids (total car), total polyphenols (TP), total flavonoids (TF), total phenolic acid (TPA), amentoflavone (a), bilobetin (b), ginkgetin (g), isoginkgetin (i), sciadopitisyn (s), total biflavones (TB).

A strong positive correlation (blue) was observed between green pigments, and between both individual and total biflavonoids, as well as between total polyphenols and biflavonoid levels. This confirms that biflavonoids are a significant group of polyphenols in ginkgo leaves, whose seasonal increase contributes to the overall rise in total polyphenols. Despite being less studied than other polyphenols [3], biflavonoids play a key role in ginkgo. Interestingly, compared to other flavonoids, biflavonoids exhibit weaker antioxidant activity, as recently reported [8]. This is further supported by the correlation matrix, which shows a negative correlation (red) between DPPH antioxidant activity and both individual and total biflavonoid content. At growth stages where biflavonoid accumulation was higher, antioxidant activity was lower. Antioxidant activity, as measured by DPPH, was instead positively correlated with total phenolic acid content, with both parameters peaking early in the season.

A negative correlation was observed between total and individual biflavonoids and chlorophyll *a*, chlorophyll *b*, and total chlorophylls. This suggests that higher chlorophyll content (as seen in Table 1 and Figure 3) is associated with lower biflavonoid levels, possibly indicating a role for biflavonoids in leaf senescence. Although the exact function of biflavonoids in plants remains unclear, there are some indications about their involvement

in photosynthesis inhibition. For instance, a study using spinach chloroplasts demonstrated that biflavonoids isolated from *Selaginella lepidophylla* inhibit ATP synthesis and other key photosynthetic processes, including electron transport, Photosystem II (PSII), Photosystem I (PSI), and their partial reactions within chloroplasts [43]. Additionally, the localization of biflavonoids in plant tissues exposed to the external environment [13] and in the outer regions of photosynthetic organs [44,45] may suggest their potential role in photosynthesis, though these hypotheses require further confirmation.

3. Materials and Methods

3.1. Chemicals, Reagents, and Instruments

To prepare the ginkgo extracts, we used 96% ethanol obtained from GRAM-MOL (Zagreb, Croatia). The following chemicals were used for phenolic component analysis: gallic acid (98%, Acros Organics, China), Folin–Ciocalteu reagent (Sigma Aldrich, Buchs, Switzerland), sodium carbonate (T.T.T., Sveta Nedelja, Croatia), (+)-catechin hydrate (Sigma Aldrich, St. Louis, MO, USA), aluminum chloride hexahydrate (Thermo Fischer Scientific, Kandel, Germany), hydrochloric acid (36.5%, Kemika, Zagreb, Croatia), sodium hydroxide (T.T.T., Sveta Nedelja, Croatia), sodium nitrite (Kemika, Zagreb, Croatia), sodium molybdate (VI) dihydrate (Sigma Aldrich, St. Louis, MO, USA), and caffeic acid (≥98%, HPLC grade, Sigma Aldrich, St. Louis, MO, USA).

All biflavonoid standards, including amentoflavone, ginkgetin, isoginkgetin, bilobetin, and sciadopitysin, were procured from PhytoLab (Vestenbergsgreuth, Germany, HPLC grade). Acetonitrile (VWR Chemicals, Radnor, PA, USA), formic acid (98–100%, Sigma Aldrich, Darmstadt, Germany), and ultrapure water (ELGA LabWater, Wycombe, UK) were used to prepare the mobile phases. For measuring antioxidant activity, we used 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma Aldrich, Steinheim, Germany), methanol (Kemika, Zagreb, Croatia), and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (97%, Sigma Aldrich, Buchs, Switzerland). Pigment determination was carried out using an acetone extract prepared with acetone from GRAM-MOL (Zagreb, Croatia).

The ginkgo leaves were dried using a freeze dryer (LIO-5PLT, KAMBIČ, Ljubljana, Slovenia), and then ground into a fine powder using a bead mill (Bead Ruptor 12, Omni International, Kennesaw, GA, USA). Ginkgo powder samples and reagent quantities for chemical preparations were accurately weighed on an analytical balance (Adam Equipment, Maidstone, UK). The extraction process was facilitated by an ultrasonic bath, a mechanical rotator (Bio RS-24, Biosan, Riga, Latvia), and a vortex mixer (V-1 plus, Biosan, Riga, Latvia). Supernatant separation was achieved via centrifugation (LMC-4200R, Biosan, Riga, Latvia). Spectrophotometric analyses were performed using a UV-VIS spectrophotometer (ONDA TOUCH UV-21, China), while biflavonoid quantification was carried out with an Agilent 1260 Infinity II high-performance liquid chromatography (HPLC) system (Agilent, Santa Clara, CA, USA) equipped with a diode array detector (DAD).

3.2. Plant Material and Sample Preparation

Ginkgo leaves were collected monthly throughout the growing season (from May to November) in 2022 from an alley of five ginkgo trees (46°09′20″ N; 16°49′46″ E) in an urban area in Koprivnica, Croatia. All trees were 30 years old, had a height of around 8 m, and were uniformly spaced 6–8 m apart. To ensure consistent leaf collection, leaves were gathered from each tree at a height of 2 to 2.5 m above the ground, with similar collection methods applied across all five tree sites. Each ginkgo tree represented a separate biological replicate. After collection, the leaves were immediately stored in a freezer at −80 °C until lyophilization. The lyophilization process was carried out for 48 h in a freeze dryer at approximately −102 °C and under pressures up to 0.3303 mBar. Once lyophilized, the leaves were ground into a fine powder using a bead mill, and this powder was then used for subsequent analyses.

3.3. Determination of Pigments

Chlorophyll pigments and carotenoids in the ginkgo leaf extract were quantified using the method described by Lichtenthaler and Buschmann [46]. Ten milligrams of the powdered sample were mixed with one milliliter of pure acetone, then centrifuged to separate the supernatant. Absorbance measurements of the extract were taken at three wavelengths (661.6 nm, 644.8 nm, and 470 nm). Specific equations, based on the solvent used, were applied to determine the concentrations of the pigments. All measurements were performed in triplicate, and results are expressed in mg per gram of dry weight (dw).

3.4. Determination of Total Polyphenols, Total Flavonoids, and Phenolic Acids

The preparation of ginkgo extract for determining total polyphenols, flavonoids, phenolic acids, and biflavonoids was conducted as follows: sixty milligrams of the powdered sample were mixed with two millilitres of 70% ethanol. The mixture was briefly vortexed and then subjected to ultrasonic treatment for 10 min at room temperature. Following this, the samples were placed on a rotary mixer for 45 min. Finally, the samples were centrifuged at 4000 RPM for 10 min, and the supernatant was collected for further analysis.

Measurements were conducted as previously reported [36]. Total polyphenols were determined using the Folin–Ciocalteu method [47], total flavonoids were quantified using the modified colorimetric method of Zhishen et al. [48], and total phenolic acids were measured using Arnow's reagent method [49]. All analyses were performed in triplicate. The concentrations of phenolic compounds in the samples were calculated from standard curves and expressed as μg mg^{-1} of dry weight (dw).

3.5. Determination of 3′,8″-Biflavones with HPLC-DAD

To determine the 3′,8″-biflavones in ginkgo leaves (amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin), we employed a slightly modified method based on Kovač Tomas et al. [13], using an Agilent 1260 Infinity II high-performance liquid chromatography (HPLC) system (Agilent, Santa Clara, CA, USA) with a diode array detector (DAD). First, standards were prepared as stock solutions at a concentration of 1 mg/mL in pure DMSO, then diluted with methanol to create working solutions at concentrations of 1, 10, 50, and 100 μg mL^{-1}. Each concentration was prepared in triplicate. Compound separation was achieved using a Zorbax 300Extend-C18 column (Agilent, Santa Clara, CA, USA) at 40 °C. The mobile phases were 0.1% formic acid solution (mobile phase A) and acetonitrile (mobile phase B), with a flow rate of 1 mL min^{-1} over a 45 min analysis period. The gradient elution profile was as follows: 0 min 98% A, 10 min 79% A, 15 min 77% A, 20 min 75% A, 25 min 64% A, 30 min 62% A, 35 min 51% A, 40 min 25% A, 43 min 8% A, and 45 min 98% A. Sample and standard injections were 10 μL each. Before injection, samples were filtered through a 45 μm pore size polytetrafluoroethylene syringe filter. Data processing was performed using Agilent OpenLab CDS software (version 2.6). Biflavone identification was achieved by comparing sample spectra with those of the standards. Chromatograms were recorded at 330 nm. The concentrations of 3′–8″ biflovones in the samples were calculated from standard curves and expressed as μg mg^{-1} of dry weight (dw).

3.6. Antioxidant Activity

Antioxidant activity was assessed using the modified Brand-Williams method [50] using the DPPH (1,1-diphenyl-2-picrylhydrazyl), as we reported previously [34]. A total of 5 μL of 70% ethanolic extract was mixed with 980 μL of 0.094 mM methanolic DPPH solution. After one hour, the absorbance was measured at 515 nm, and the results were expressed as Trolox Equivalents per gram of dry weight (μmol TE g^{-1} dw).

3.7. Statistical Analysis

The sampling of leaves was conducted from five ginkgo trees which represent five biological replicates. All measurements were performed at least in triplicate, and results are presented as the mean value ± standard deviation (SD). Statistical data analysis was

conducted using PAST software (version 4.15), employing one-way ANOVA and post hoc Tukey's test. Differences between samples were considered significant at $p < 0.05$

4. Conclusions

We collected ginkgo leaves at seven developmental stages throughout the season, from May to November, and assessed chlorophyll content, carotenoids, total polyphenols, total flavonoids, total phenolic acids, and antioxidant activity spectrophotometry. Additionally, we measured the content of 3′,8″-biflavones using HPLC-DAD. Throughout the season, we observed increases in total carotenoid content, total polyphenols, flavonoids, and total 3′,8″-biflavones, including individual biflavonoids such as bilobetin, ginkgetin, isoginkgetin, and sciadopitysin. Amentoflavone was the least abundant and did not increase over the season. Sciadopitysin was the most abundant biflavonoid at all stages. We also detected a negative correlation between biflavonoid and chlorophyll content, suggesting that biflavonoids may be involved in leaf senescence. This potential role warrants further investigation.

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Data Availability Statement: Data are contained within the article.

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5.2. Additional articles

5.2.1. Influence of Air Drying, Freeze Drying and Oven Drying on the Biflavone Content in Yellow Ginkgo (*Ginkgo biloba* L.) Leaves

applied sciences

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Article

Influence of Air Drying, Freeze Drying and Oven Drying on the Biflavone Content in Yellow Ginkgo (*Ginkgo biloba* L.) Leaves

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Abstract: Drying herbs is a crucial method for stabilizing and preserving their essential properties and bioactive compounds. Although freeze drying is the preferred method for most herbs, it is expensive due to high energy consumption and operating costs. Biflavonoids are dimeric flavonoids that have recently been recognized as potential molecules possessing biological activities, such as antiviral and antimicrobial activity, and as effective molecules for the treatment of neurodegenerative and metabolic diseases and for cancer therapies. In this study, we performed a comparative analysis of freeze drying, air drying and oven drying to evaluate their effects on biflavonoid content in yellow ginkgo leaves (*Ginkgo biloba* L.). After drying, we performed spectrophotometric analysis to determine the browning index, pigments, phenolic compounds and antioxidant activity, while HPLC-DAD was used for the identification and quantification of individual biflavones (amentoflavone, bilobetin, ginkgetin, isoginkgetin and sciadopitysin). The most abundant biflavonoids were isoginkgetin and bilobetin, the amounts of which exceeded 1000 μg/g dw in all leaf samples. They were followed by ginkgetin and sciadopitysin, the amounts of which were about 30% lower. The drying method did not influence biflavone content or the total carotenoids, total polyphenols and total flavonoids. Consequently, our study suggests that all three methods may be used for the preparation of yellow ginkgo leaves as a source of biflavones and other bioactive compounds.

Keywords: air drying; biflavonoids; freeze drying; ginkgo; oven drying

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1. Introduction

The ginkgo (*Ginkgo biloba* L.) is the only member of the Ginkgoaceae family whose fossil remains are over 200 million years old, earning it the nickname "living fossil" [1]. It is characterized by a distinctive crown and trunk, which is most notable for its unique fan-shaped leaves with dichotomously branched veins. This species originates from southeast China and its cultivars are now widespread throughout the world [2]. Ginkgo is often associated with Eastern medicinal and religious traditions and has attracted considerable interest due to its beneficial health effects, particularly in relation to improving memory and the cardiovascular system [3]. These properties are primarily attributed to various bioactive compounds in ginkgo leaves such as flavonoids, phenolic acids and terpenoids [4]. More than a hundred flavonoids have been identified in ginkgo, which occur in two forms—as monomers and dimers in the form of aglycones or glycosides [4]. Biflavonoids, the dimeric forms of flavonoids, are commonly found in ginkgo leaves [5]. They include amentoflavone, bilobetin, ginkgetin, isoginkgetin and sciadopitysin as the predominant biflavones [5,6]. Biflavonoids have recently become of interest due to their antiviral, anticarcinogenic and neuroprotective effects [6,7]. In particular, they are known as antiviral agents against the COVID-19 virus [8,9]. Yellow ginkgo leaves are particularly rich in the biflavones amentoflavone, bilobetin, ginkgetin, isoginkgetin and sciadopitysin, the content of which is higher in yellow leaves than in green leaves [10].

Drying of herbs is a crucial technological process primarily aimed at removing the water content and enabling longer preservation of raw materials [11]. The drying process of plant material facilitates the removal of water, effectively suppresses metabolic processes and ensures the stability of the chemical composition of the plant material [11]. In addition, it helps to minimize the presence of microorganisms so that the full range of physicochemical and sensory properties of the plant product is preserved [12]. Drying processes can take place under natural conditions, such as air drying, or in controlled artificial environments with temperature control (either high or low) and vacuum. Considering the time required and the need to prevent the possible contamination or damage of samples by external factors, artificial drying systems are increasingly preferred, especially drying chambers and freeze drying [13]. As elevated drying temperatures can lead to degradation of and reductions in the desired components, freeze drying is increasingly being used [11,14]. Drying methods have a major impact on the various properties of the final product, including the presence and composition of bioactive components as well as physicochemical and organoleptic properties [15]. In high temperature drying, a hot air circulation system is used to vaporize water, while in freeze drying, also known as vacuum drying, the water is suppressed under low pressure after the plant material has been previously frozen [11]. Freeze drying allows high efficiency in removing water from the plant matrix while keeping all bioactive components stable and preserving their chemical structure. It is often the method of choice to extract flavonoids [13]. Although heat and freeze drying techniques shorten the drying time, they also have their limitations. For example, freeze drying is energy-intensive, while heat drying can affect quality [14]. The use of an appropriate drying method can therefore reduce production costs, preserve the bioactive compounds in the material and improve the quality of the final products. However, the literature shows that the effect of leaf drying depends on the type of leaves. In the case of mulberry leaves, Hu et al. [16] reported that air drying and freeze drying are the best methods to preserve the antioxidant activity of flavonoids. Since air drying has lower operating costs than freeze drying to obtain high flavonoid content and maximum antioxidant activity, they recommended air drying for mulberry leaves. In contrast, freeze drying of leaves was preferred for *Carica papaya* L. [17], *Streblus asper* Lour [18], green tea (*Camellia sinensis* L.) [19] and guava (*Psidium guajava* L.) [20] leaves to obtain the desired amounts of polyphenols and flavonoids. For nettle (*Urtica dioica* L.) leaves, the total phenol content and antioxidant activity were higher in oven-dried leaves than in freeze-dried leaves [21], while for *Anneslea fragrans* leaves [22], shade drying is the preferred drying method. This once again underlines the need to optimize the drying method for each leaf type and for each bioactive compound.

In the case of ginkgo, drying methods for ginkgo seeds have been studied [23–27], but little attention has been paid to the influence of the drying method on the bioactive compounds in ginkgo leaves. Considering the growing interest in biflavonoids and the fact that yellow ginkgo leaves are a potential source of biflavonoids [6], we wanted to test different drying methods for yellow ginkgo leaves and investigate how they affect biflavonoids and other bioactive compounds. As far as we know, this is the first report on the influence of drying method on the form of dimeric flavonoids in yellow ginkgo leaves and on biflavones overall. We compared three drying methods for plant material: freeze drying, air drying and oven drying for yellow ginkgo leaves. After drying, we determined the browning index and the total content of phenols, flavonoids, phenolic acids and pigments (chlorophyll *a*, *b*, total chlorophylls and carotenoids) spectrophotometrically and the content of the five most abundant biflavonoids in the ginkgo leaves (amentoflavone, bilobetin, ginkgetin, isoginkgetin, sciadopitysin) by HPLC-DAD. We also measured the antioxidant scavenging capacity using the DPPH method.

2. Materials and Methods

2.1. Chemicals, Reagents and Instruments

We used the following chemicals and reagents: ethanol 96% (GRAM-MOL, Zagreb, Croatia), acetone (GRAM-MOL, Zagreb, Croatia), acetonitrile ≥ 99.9% UHPLC gradient-grade (Fischer Scientific, Taipei City, Taiwan), formic acid 98–100% (Sigma Aldrich, Darmstadt, Germany), HPLC grade-standard amentoflavone (PhytoLab, Vestenbergsgreuth, Germany), ginkgetin (PhytoLab, Vestenbergsgreuth, Germany), isoginkgetin (PhytoLab, Vestenbergsgreuth, Germany), bilobetin (PhytoLab, Vestenbergsgreuth, Germany) and sciadopitysin (PhytoLab, Vestenbergsgreuth, Germany), gallic acid 98% (Acros Organics, China), Folin–Ciocalteu's reagent (Sigma Aldrich, Buchs, Schwitzerland), sodium carbonate (T.T.T., Sveta Nedelja, Croatia), (+)-catechin hydrate (Sigma Aldrich, St. Louis, MO, USA), aluminum chloride hexahydrate (Thermo Fischer Scientific, Kandel, Germany), hydrochloric acid 36.5% (Kemika, Zagreb, Croatia), sodium hydroxide (T.T.T., Sveta Nedelja, Croatia), sodium nitrite (Kemika, Zagreb, Croatia), sodium molybdate (VI) dihydrate (Sigma Aldrich, St. Louis, MO, USA), caffeic acid ≥ 98% HPLC-grade (Sigma Aldrich, St. Louis, MO, USA), 2,2-diphenyl-1-picrylhydrazyl (Sigma Aldrich, Steinheim, Germany), methanol (Kemika, Zagreb, Croatia), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid 97% (Sigma Aldrich, Buchs, Schwitzerland).

For drying samples, we used a freeze dryer (LIO-5PLT, KAMBIČ, Ljubljana, Slovenia) and oven dryer (VENTI-Line 180 Prime, VWR, Gdańsk, Poland). For grinding leaves, a bead mill (Bead Ruptor 12, Omni International, Kennesaw, GA, USA) was used. For weighing plant material, an analytical balance (Adam Equipment, Maidstone, UK) was utilized. For preparing extracts, an ultrasonic bath (DU-100, Argo Lab, Carpi, Italy), mechanical rotator (Bio RS-24, Biosan, Riga, Latvia), vortex (V-1 plus, Biosan, Riga, Latvia) and centrifuge (LMC-4200R, Biosan, Riga, Latvia) were employed. For spectrophotometric analyses, a UV-VIS spectrophotometer (ONDA TOUCH UV-21, China) was used. For determining biflavonoids, an Agilent 1260 Infinity II high-performance liquid chromatography system (Agilent, Santa Clara, CA, USA) with a diode array detector (DAD) was employed. System controlling and data analysis was performed using OpenLab CDS Workstation Software version 2.8.

2.2. Plant Material and Drying Experiments

Yellow ginkgo leaves were collected from nine different trees in a ginkgo alley in Koprivnica, Croatia in October 2022. We collected the leaves from each tree (in equal amounts, around 100 leaves). The leaves were immediately brought to the laboratory on ice and then divided into three groups for different drying methods (approximately 100 leaves for each method). This ensured that our sample sizes were homogenized and that the only parameters that could influence the differences were the different drying methods.

The plant material was then immediately subject to different drying methods: freeze drying, air drying and oven drying. All leaves were dried until a constant weight was reached. Leaves were dried in a freeze dryer (LIO-5PLT, KAMBIČ, Ljubljana, Slovenia) under conditions of approximately −102 °C and 0.3303 mBar pressure. Before freeze drying, leaves were frozen at −80 °C for two hours and then transferred to a freeze dryer already operating at freezing temperature. Air drying of the leaves was performed in a laboratory bench at room temperature around 21–23 °C and humidity 30–50% without direct sun exposure but in light for a part of the day. The third type of drying, oven drying (VENTI-Line 180 Prime, Poland) with circulating air, was carried out at 60 °C and with the air flap control set to 50%.

After reaching the constant weight (which was checked by regular leaf weighing) the dried leaves (Figure 1) were ground into fine powder using a bead mill (Bead Ruptor 12, Omni International, USA). Samples dried in a different way were ground together in one run in separated tubes with the addition of a 2.4 mm metal bead at 6 m/s for 3 min, and the resulting powder was used for all subsequent analyses.

Figure 1. Yellow ginkgo leaves and leaf powder after freeze drying, air drying and oven drying, respectively.

2.3. Browning Index

The browning index (BI) was determined according to the method described by Lee et al. [28]. Ten milligrams of the ginkgo powder were mixed with one millilitre of pure acetone and then centrifuged. The supernatant was decanted and transferred to a plastic cuvette, and the absorption was read at 420 nm. The results are given as values of the measured absorption values.

2.4. Pigments

The measurement of pigments included the quantitative determination of chlorophylls and carotenoids in tissue according to the method of Lichtenthaler and Buschmann [29]. Ten milligrams of ginkgo powder were mixed with one millilitre of pure acetone and then centrifuged. The supernatant was separated and the precipitate was discarded. The absorbance was measured in the supernatant at three wavelengths (661.6, 644.8 and 470 nm), which were used for further calculations for pure acetone as solvent. The results are given in µg/g dw.

2.5. Phenolic Compounds

For the determination of total phenols, flavonoids, phenolic acid and individual biflavonoids, 60 mg of ginkgo leaf powder was weighed and 2 ml of 70% ethanol was added. The samples were then shaken and incubated in an ultrasonic bath for 10 min. The samples were then placed in a rotator for 45 min. The supernatant was separated after centrifugation and used for the analysis.

Total polyphenols were determined by the colorimetric method using Folin–Ciocalteu (FC) reagent [30]. First, 200 µL of the extract was mixed with 1580 µL of distilled water and 100 µL of FC reagent. Then, 300 µL of sodium carbonate was added and after two hours the absorbance was measured at 765 nm. The standard curve was prepared with gallic acid and the results are expressed as gallic acid equivalents per dry weight (µg GAE/mg dw). The determination of total flavonoids was performed according to the method of Zhishen et al. [31]. Initially, 200 µL of the extract was mixed with 800 µL of distilled water. The reaction was started with 60 µL $NaNO_2$ (5% solution). After five minutes, 60 µL of a 10% solution of Al_2Cl_3 was added. After a further 6 min, 400 µL NaOH (1 M) was added and the absorbance was measured at 510 nm. The standard curve was prepared with catechol and the results are expressed as catechol equivalents per dry weight (µg CAE/mg dw). Total phenolic acids were determined using Arnow's reagent method [32]. Initially, 300 µL of the extract was mixed with 300 µL distilled water, 100 µL HCl (0.5 M) and 100 µL Arnow's reagent. Subsequently, 100 µL NaOH (1 M) and 100 µL distilled water were added. The absorbance was measured at 505 nm. The standard curve was prepared with caffeic acid and the results were expressed as caffeic acid equivalent per dry weight (µg CA/mg dw).

Identification and quantification of the individual bioflavonoids (amentoflavone, bilobetin, ginketin, isogingetin and sciadopitysin) was performed using an Agilent 1260 Infinity II high-performance liquid chromatography system (Agilent, Santa Clara, CA, USA) with a diode array detector (DAD). Chromatographic separation was performed using a Zorbax 300Extend-C18 column (Agilent, Santa Clara, CA, USA). Data acquisition and subsequent processing were performed using Agilent OpenLAB CDS software (version 2.6, Agilent, Santa Clara, CA, USA). The extraction, analysis parameters and calibration curves were consistent with our previously published work by Kovač Tomas et al. [5]. The results were expressed as μg/g dw.

2.6. Antioxidant Activity

Antioxidant activity was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay [33]. A total of 5 μL of the ethanolic extract was mixed with 995 μL of the DPPH solution and the mixture was allowed to stand in the dark for about half an hour. The absorbance was measured at 515 nm. The standard curve was prepared with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and so the results are expressed as Trolox equivalents per dry weight (mM TE/g dw).

2.7. Statistical Analysis

All analyses were conducted in triplicate, and the results are expressed as mean ± standard deviation (SD). All statistical analyses were performed using PAST software, version 4.13. One-way ANOVA and subsequent multiple mean comparisons with Tukey's HSD test were executed, and distinctions between measurements were considered significant at $p < 0.05$.

3. Results

3.1. Influence of Different Drying Methods on the Browning Index

The influence of different drying methods—freeze drying, air drying and oven drying—on the browning index is shown in Figure 2.

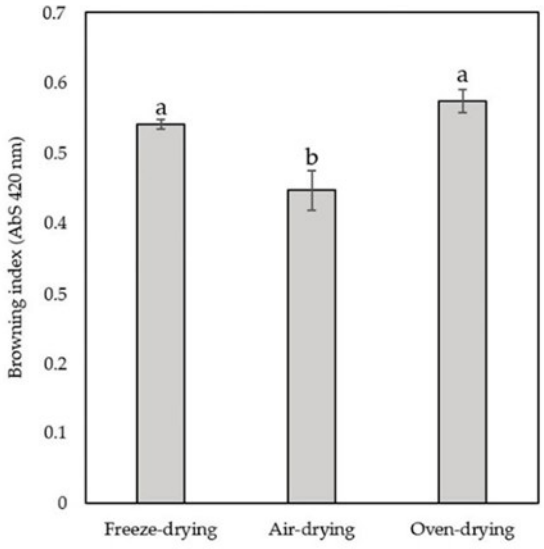

Figure 2. Browning index for the three types of drying yellow ginkgo leaves. Values marked with different letters are significantly different at $p < 0.05$.

Oven-dried (0.57 ± 0.02) and freeze-dried (0.54 ± 0.01) leaves showed comparable values, while the significantly lowest values were observed for air-dried leaves (0.45 ± 0.03).

3.2. Influence of Different Drying Methods on the Composition of Chlorophylls and Carotenoids

The content of chlorophyll *a* and *b*, total chlorophylls and carotenoids, as well as the ratios of chlorophyll *a*/*b* and total chlorophylls to carotenoids in yellow ginkgo leaves dried using three different methods are presented in Table 1.

Table 1. The content of pigments in yellow ginkgo leaves affected by different drying methods. Values marked with different letters are significantly different at $p < 0.05$.

	Freeze Drying	Air Drying	Oven Drying
Chlorophyll *a* (μg/g dw)	64.40 ± 2.03 ^{b}	20.32 ± 0.95 ^{c}	86.27 ± 3.12 ^{a}
Chlorophyll *b* (μg/g dw)	22.78 ± 1.52 ^{b}	15.15 ± 0.93 ^{c}	30.74 ± 3.59 ^{a}
Total chlorophylls (μg/g dw)	87.18 ± 2.81 ^{b}	35.47 ± 1.86 ^{c}	117.01 ± 6.54 ^{a}
Total carotenoids (μg/g dw)	174.78 ± 8.04 ^{a}	164.60 ± 5.81 ^{a}	159.8 ± 6.03 ^{a}
Chlorophyll *a*/Chlorophyll *b*	2.84 ± 0.20 ^{a}	1.34 ± 0.03 ^{b}	2.84 ± 0.26 ^{a}
Chlorophylls/carotenoids	0.50 ± 0.01 ^{b}	0.22 ± 0.01 ^{c}	0.73 ± 0.02 ^{a}

According to the data presented in Table 1, the highest content of chlorophyll *a* and *b* and total chlorophyll was observed in the samples dried by oven drying, followed by freeze drying and air drying. However, the total carotenoid content showed no statistical differences between the three drying methods and all leaf samples showed values in the range of 159–174 μg/g dw.

The ratio of chlorophyll *a* to chlorophyll *b* was lower in the air-dried samples and comparable in the freeze-dried and oven-dried samples. Similarly, the chlorophyll/carotenoid content was also much lower in air-dried leaves. The low chlorophyll/carotenoid content indicates a higher carotenoid content, which is to be expected in yellow leaves as we used in this experiment.

3.3. Influence of Different Drying Methods on the Content of the Total Polyphenols, Flavonoids, Phenolic Acids and Individual Biflavonoids

The influence of the different drying methods on the content of total polyphenols, total flavonoids and total phenolic acids is shown in Figure 3.

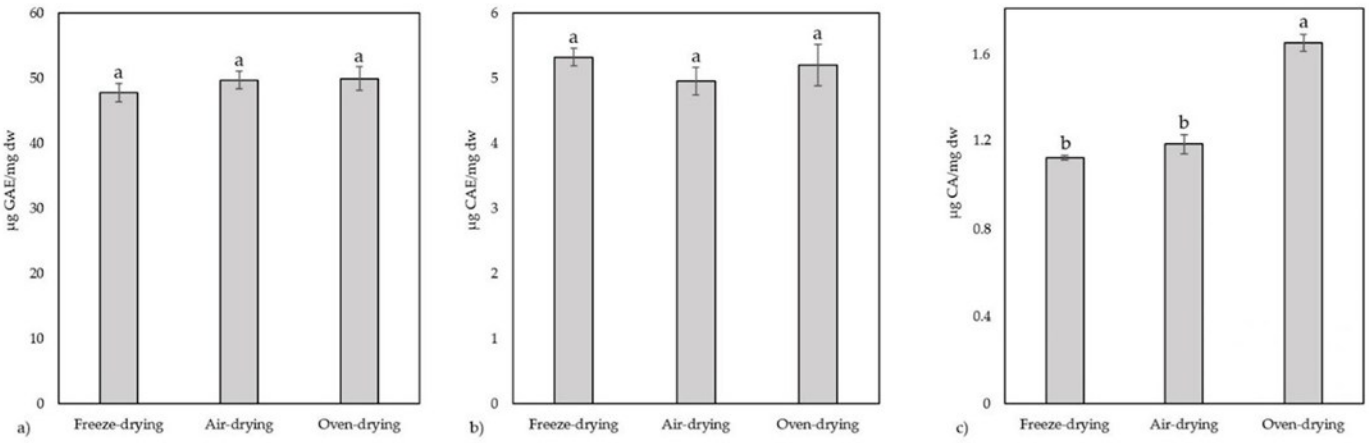

Figure 3. Total polyphenols (**a**), total flavonoids (**b**) and total phenolic acids (**c**) for the three types of drying used on yellow ginkgo leaves. Values marked with different letters are significantly different at $p < 0.05$.

The total content of polyphenols and flavonoids did not differ significantly in the leaf samples dried by different methods. Non-significantly, the highest levels of total flavonoids were found in the freeze-dried samples (5.32 ± 0.13 μg CAE/mg dw), while the oven-dried samples had the highest levels of total polyphenols (49.92 ± 1.81 μg GAE/mg dw).

Oven-dried leaves had the highest content of total phenolic acids (1.64 ± 0.04 µg CA/mg dw), and this value was significantly higher.

The influence of different drying methods on the individual composition of biflavonoids in yellow ginkgo leaves is shown in Figure 4.

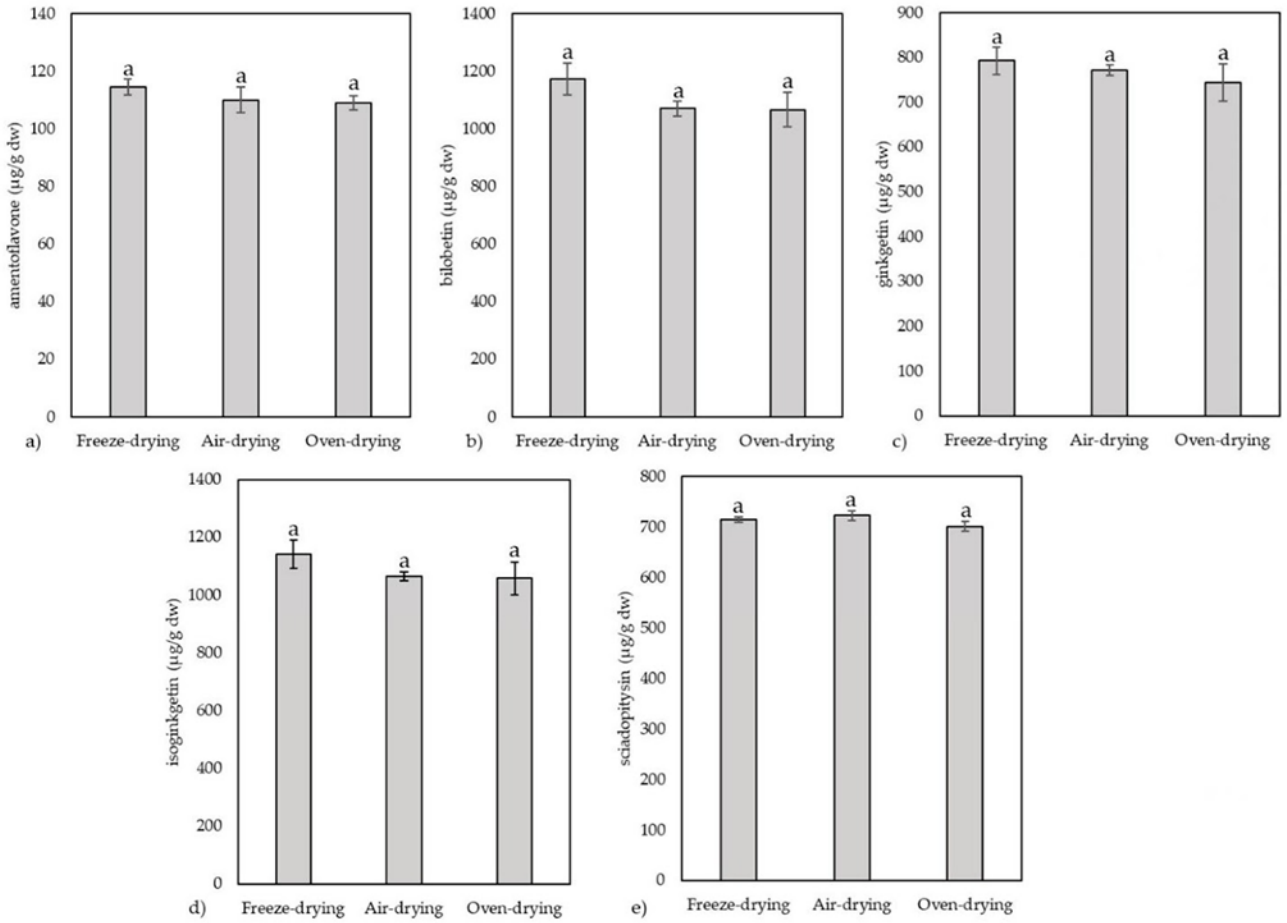

Figure 4. The individual biflavonoid composition, including amentoflavone (**a**), bilobetin (**b**), ginkgetin (**c**), isoginkgetin (**d**) and sciadopitysin (**e**), for the three types of drying used on yellow ginkgo leaves. Values marked with different letters are significantly different at $p < 0.05$.

The most abundant biflavonoids were isoginkgetin and bilobetin, the amount of which exceeded 1000 µg/g dw in all leaf samples. They were followed by ginkgetin and sciadopitysin, the amounts of which were about 30% lower, in the range of 700–800 µg/g dw. The least abundant was amentoflavone, which was just over 100 µg/g dw, 10 times less than isoginkgetin and bilobetin. Freeze-dried samples showed slightly higher average values, but these were not significant, so our results indicate that the drying method had no significant effect on the content of individual biflavonoids.

3.4. Influence of Different Drying Methods on Antioxidant Activity

The influence of the different drying methods on the antioxidant activity of the yellow ginkgo leaves is shown in Figure 5.

The radical scavenging capacity ranged from 50.13 ± 5.98 mM TE/g dw in air-dried samples to 54.03 mM TE/g dw in freeze-dried samples. Nevertheless, there were no statistically significant differences depending on the drying method.

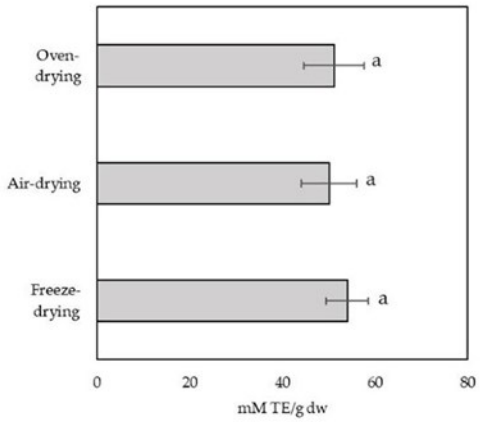

Figure 5. Antioxidant activity analysed by DPPH for the three types of drying used on yellow ginkgo leaves. Values marked with different letters are significantly different at $p < 0.05$.

4. Discussion

During drying, undesirable colour changes may occur in dried plant materials due to non-enzymatic browning, which may affect consumer preference for the products, but also decrease bioactive compounds [34]. Therefore, we compared the browning index of our samples and found a comparable value in the oven-dried and freeze-dried samples, while the browning index was lowest in the air-dried samples. In general, according to our results, all three methods did not lead to the formation of brown pigments in large quantities. In contrast, for pine needles, hot air drying and freeze drying were reported to increase the browning index four-fold compared to freeze drying [35]. Yilmaz and Alibas [36] reported that higher drying temperature leads to a higher browning index in basil leaves, which is consistent with our results, in which air-dried samples without high temperature had the lowest browning index compared to the oven-dried ones.

The colour of the leaves is also influenced by pigments. Green leaves are dominated by chlorophylls, the amount of which decreases as the leaves age, and yellow leaf colour is due to the presence of carotenoids (yellow pigments) or other flavonoids, depending on the leaf [37]. Although we used yellow leaves, they still contained some chlorophylls, as can be seen from the results in Table 1. The ratio between total chlorophyll and total carotenoids was low, indicating a low chlorophyll content, which is characteristic of yellow leaves. According to our results, the oven-dried samples had significantly higher chlorophyll *a*, *b* and total chlorophyll content compared to the air-dried or freeze-dried samples. It is known that chlorophylls are sensitive to light, so these results were to be expected. The air-dried samples were exposed to light, as were the freeze-dried samples, as the design of our freeze-dryer allows the samples to be exposed to light. In contrast, the samples in the oven were protected from light and had the highest chlorophyll content. Similar to our experiments, Shittu et al. [38] reported significantly lower chlorophyll content in air-dried (in the shade) than in heat-dried mint leaves. In our experiment, the chlorophyll *a*/chlorophyll *b* ratio was lowest in the air-dried samples, which is probably due to the rapid degradation of chlorophyll *a*. A similar finding was observed in peppermint leaves, where a lower chlorophyll *a*/chlorophyll *b* ratio was found in leaves dried at a temperature of 22 ± 2 °C in air than in leaves dried with hot air or infrared, convection or microwave dryers [39]. In our experiments, all drying methods had an effect on the green chlorophylls, but in contrast the carotenoids were stable and their content did not differ between the samples dried by different methods. Carotenoids are associated with numerous biological activities, such as anticancer, immunomodulatory, anti-inflammatory, antibacterial, antidiabetic and neuroprotective activities [40].

From the point of view of potential pharmaceutical use, the important compounds in ginkgo leaves are those from the polyphenol group [4]. The total polyphenol content in our samples was about 50 µg GAE/mg dw and did not differ significantly from the leaves dried

in different ways. The measured amount is higher than in other medicinal plants, such as *Hypericum perforatum* L. [41,42], *Gynostemma pentaphyllum* L. [43] or *Micromeria croatica* (Pers.) Schott [44], which were previously measured using similar protocols. The total flavonoid content was also not significantly different in the ginkgo samples and was around 5 μg CE/mg live weight, while the total phenolic acid content was highest in the oven-dried samples and lowest in the freeze-dried and air-dried samples. The higher amount in the oven-dried samples could be due to the higher temperature, which is reported as a favourable parameter for the extraction of phenolic acids [45]. We also performed quantification of individual biflavonoids and identified five biflavonoids, similar to a previous study on ginkgo leaves [5]. We identified isoginkgetin and bilobetin as the most abundant biflavonoids in yellow ginkgo leaves, which is consistent with previously published studies [46]. Isoginkgetin is known as a general pre-mRNA splicing inhibitor and, as such, has been implicated in research as a new avenue for the development of novel anticancer drugs [47]. Our results show that yellow ginkgo leaves may be an important source of isoginkgetin. Isoginkgetin has a yellow colour, as does ginkgetin, so the characteristic yellow colour of the leaves is probably also due to the presence of biflavonoids. The content of individual biflavonoids in ginkgo leaves may differ at different stages of development or be influenced by growing conditions and other environmental conditions (reviewed by [6]). According to our study, the drying method had no effect on the biflavonoid content, suggesting that air drying can also be used as an alternative to more expensive methods for drying yellow ginkgo leaves as a source of biflavonoids.

Ginkgo leaf extracts are generally reported to have antioxidant activity [48,49], and according to our results, the antioxidant activity measured by the DPPH method was not affected by the drying method. This is probably due to the similar content of polyphenols and flavonoids in the leaves dried by different methods, which are recognized as potent antioxidants [50]. However, our recent study showed that biflavonoids are less potent antioxidants than monomeric flavonoids [51], but their antioxidant activity remains contradictory.

5. Conclusions

In this study, we investigated the effects of three different drying methods on the browning index, pigment content, polyphenolic components, especially biflavonoids, and antioxidant activity. The most abundant biflavonoids in yellow ginkgo leaves are isoginkgetin and bilobetin. The drying methods affected the browning index, chlorophyll content and total phenolic acid content, while the total carotenoid content, total polyphenols, total flavonoids and individual biflavonoids remained the same. This work represents the first comparison of different drying methods on the individual composition of biflavonoids in yellow ginkgo leaves, one of the major specialized metabolites contributing to the medicinal properties of ginkgo. Our results suggest that any of the three drying methods can be used for drying ginkgo leaves, maximizing the preservation of biflavonoids.

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5.2.2. Neuroprotective, Anti-Hyperpigmentation, and Anti-Diabetic Effects and Bioaccessibility of Flavonoids in Ginkgo Leaf Infusions from Green and Yellow Leaves

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Neuroprotective, Anti-Hyperpigmentation, and Anti-Diabetic Effects and Bioaccessibility of Flavonoids in Ginkgo Leaf Infusions from Green and Yellow Leaves

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Abstract: Ginkgo (*Ginkgo biloba* L.) is a widely used medicinal plant, with its green spring leaves commonly utilized for preparing extracts with various therapeutic properties, and leaf infusions also frequently employed. This study aimed to evaluate the in vitro neuroprotective, anti-hyperpigmentation, anti-diabetic, and antioxidant activities, as well as the flavonoid content and its bioaccessibility, of ginkgo leaf infusions, comparing leaves collected in spring and autumn. Infusions made from yellow leaves, both those collected directly from the tree and fallen leaves, exhibited significantly higher total polyphenol content (3.2-fold and 2.5-fold, respectively) and flavonoid content (3.1-fold and 2.4-fold, respectively), along with greater flavonoid bioaccessibility in the salivary phase. These infusions also demonstrated enhanced tyrosinase inhibition (6.0-fold and 5.7-fold, respectively) and antioxidant activity (4.8-fold and 3.5-fold, respectively). Notably, infusions from fallen yellow leaves showed 2.5-fold higher acetylcholinesterase inhibition compared to spring leaf infusions, while α-glucosidase inhibition remained comparable across all samples. These findings suggest that yellow ginkgo leaves, including those that have fallen, could be considered a valuable material for making infusions with potential neuroprotective, anti-hyperpigmentation, and anti-diabetic properties.

Keywords: ginkgo; infusions; biological activity; polyphenolics

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1. Introduction

Ginkgo (*Ginkgo biloba* L.), one of the oldest living tree species, has a long history of use in traditional medicine, particularly in China, where it has been employed for over a thousand years to treat ailments such as asthma, bronchitis, and memory loss [1]. The leaves and seeds of ginkgo were used to improve circulation and lung function, as well as to enhance mental clarity. In modern medicine, ginkgo is widely recognized for its neuroprotective properties, especially in managing cognitive disorders like Alzheimer's disease and dementia [2]. Its rich content of flavonoids and terpenoids provides antioxidant benefits, helping to reduce oxidative stress and improve blood flow, defending against cancers and protecting nerve cells from damage [3,4]. Today, ginkgo supplements are commonly available in various forms such as capsules, extracts, and infusions, bridging traditional knowledge with modern pharmacology.

Ginkgo infusions, made from the leaves of the ginkgo tree, are popular for their potential health benefits, particularly in enhancing cognitive function and improving circulation [1]. Drinking ginkgo tea may boost memory, concentration, and mood, while also offering relief for conditions like peripheral artery disease, tinnitus, and migraines. Ginkgo infusions, commonly also called ginkgo teas, possess antioxidant capacity and contain polyphenolic compounds with antioxidant activity [5–7]. Flavonoids, ginkgolides, and aromatic components have also been identified in ginkgo teas [8]. In vitro studies showed that ginkgo infusions possess cytotoxicity on HepG2 cells [7].

In general, the bioactivity of infusions may be influenced by factors such as the plant species and the environmental conditions in which the plants are grown, the specific plant part used for the infusion, as well as the drying method of the plant material [9]. Additionally, the preparation protocol, including water temperature and infusion time, can also impact the bioactive properties [9]. The bioactivity of ginkgo extracts is influenced by the tree's growing location and season, as well as the solvent used in the extraction process [10]. Guo et al. [11], who performed a metabolomics study of ginkgo leaves collected in June and September, reported that the developmental stage is a critical factor in specialized metabolite variations. According to Pereira et al. [12], ginkgo infusions contain fewer bioactive compounds and have a potentially lower bioactivity compared to extracts prepared in organic solvents (due to the reduced solubility of certain compounds in water). In another study, ginkgo leaf infusions had higher total phenolic content than ethanolic extracts [13]. In any case, the ease of preparation and wide availability of infusions make them a popular choice for consumers globally. The drying method of ginkgo leaves does not affect their polyphenolic compounds or antioxidant activity [14]. However, the processing methods used to prepare ginkgo leaf tea, such as white tea, black tea, dark tea, and green tea, significantly influence its bioactivity [7]. According to Goh et al. [5], an infusion temperature of 100 °C and infusion time around 10–15 min gave the highest overall antioxidant capacity.

Ginkgo infusions are typically prepared using young green leaves. However, some studies have reported that yellow ginkgo leaves may also contain a significant amount of bioactive compounds [14], potentially even more than the green leaves [13,15]. This suggests that leaves at different developmental stages may offer unique benefits for infusion preparation. However, there is a lack of studies directly comparing the bioactivity of infusions made from leaves at various stages of development. Thus, we assessed the total polyphenolic and flavonoid content, as well as the in vitro antioxidant, neuroprotective, anti-hyperpigmentation, and anti-diabetic activities and flavonoid bioaccessibility of ginkgo infusions prepared from both green leaves collected in spring and yellow leaves collected in autumn. Ginkgo trees are known for shedding most of their leaves simultaneously. In addition to yellow leaves which we collected from the tree, we collected healthy leaves immediately after they had fallen to assess their biological potential. Harvesting fallen leaves instead of picking them directly from the tree could reduce labor costs and minimize damage to the tree, making it a more sustainable method for leaf collection.

2. Materials and Methods

2.1. Plant Material and Infusions Preparation

Ginkgo leaves were harvested from a ginkgo tree valley (46°09′20″ N; 16°49′46″ E) in Koprivnica, Croatia in May and October 2022. In October, healthy leaves that had naturally fallen from the trees were collected shortly after they dropped. The leaves were stored at −80 °C until further use. The leaves were lyophilized in a freeze-dryer (LIO-5PLT, KAMBIČ, Ljubljana, Slovenia) for 48 h at a temperature of around −102 °C and pressures up to 0.3303 mBar. After lyophilization, the ginkgo leaves were manually crushed in a bag to make them suitable for preparing herbal infusions.

On an analytical balance (Adam Equipment, Maidstone, UK), 20 mg each of spring, autumn, and fallen autumn leaves were weighed in triplicate. Two milliliters of boiling water were added to each test tube. The samples were placed on a circular rotator (Bio RS-24, Biosan, Riga, Latvia) for 15 min. Afterwards, the resulting herbal leaf infusions were separated from the sediment by decanting into new test tubes and were used for further analyses.

2.2. Determination of Total Flavonoid and Total Polyphenol Content

The total polyphenol content was determined using a modified Folin–Ciocalteu method [16]. First, 20 µL of the ginkgo leaf extract was combined with 1580 µL of distilled water. Then, 100 µL of Folin–Ciocalteu reagent and 300 µL of a saturated sodium carbonate

solution were added, and the mixture was thoroughly stirred. The solution was allowed to incubate at room temperature for two hours before measuring absorbance (ONDA TOUCH UV-21, Shanghai, China) at 765 nm. A standard curve was prepared using gallic acid, and polyphenol content in the samples was reported as gallic acid equivalents per milligram of dry weight (μg GAE/mg dw).

The total flavonoid content was measured using a modified colorimetric method based on Zhishen et al. [17]. In this procedure, 200 μL of ginkgo leaf infusion was mixed with 800 μL of distilled water. The reaction began with the addition of 60 μL of a 5% sodium nitrite solution. After five minutes, 60 μL of a 10% aluminum chloride solution was introduced, followed by 400 μL of a 1 M sodium hydroxide solution after six minutes. The mixture was then topped up with 480 μL of distilled water and thoroughly mixed. A calibration curve using catechin as the standard was prepared, and flavonoid levels in the samples were expressed as micrograms of catechin equivalents per milligram of dry weight (μg CE/mg dw).

2.3. Determination of Flavonoid Bioaccessibility

An in vitro digestion model [18], based on Vujčić Bok et al. [19], was used to study the bioaccessibility of flavonoids from infusions prepared from green and yellow leaves. The process included an initial phase, a salivary phase with amylase addition, followed by simulated gastric digestion using porcine pepsin and HCl. After adjusting the pH, an intestinal phase was mimicked by adding pancreatic juices. The final digestion step involved incubation and pH adjustment to 7, followed by centrifugation. Supernatants were analyzed by previously reported HPLC–DAD method [20] with slight modifications. In summary, an Agilent 1260 Infinity II high-performance liquid chromatography (HPLC) system (Agilent, Santa Clara, CA, USA) equipped with a diode array detector (DAD) was utilized. The separation of components was performed using a Zorbax 300 Extend-C18 column (Agilent, Santa Clara, CA, USA) at 40 °C. The mobile phase consisted of a combination of phase A—0.1% formic acid solution—and phase B—acetonitrile, with a flow rate of 1 mL/min over a 45 min period. The mobile phase gradient was as follows: 0 min, 98% A; 10 min, 79% A; 15 min, 77% A; 20 min, 75% A; 25 min, 64% A; 30 min, 62% A; 35 min, 51% A; 40 min, 25% A; 43 min, 8% A; and 45 min, 98% A. Ginkgo infusions were filtered through 45 μm PTFE syringe filters. Standards of biflavonoids were used to prepare calibration curves, and chromatograms were recorded at 330 nm. A representative chromatogram is available in the Supplementary Materials. To determine flavonoid bioaccessibility, we recorded the area under all peaks at 330 nm. The accessibility during each specific digestion phase was calculated as follows [19]:

$$\text{Bioaccessibility in salivary phase} = (\text{BA}_{\text{salivary phase}}/\text{BA}_{\text{initial phase}}) \times 100$$

$$\text{Bioaccessibility in gastric phase} = (\text{BA}_{\text{gastric phase}}/\text{BA}_{\text{initial phase}}) \times 100$$

$$\text{Bioaccessibility in intestinal phase} = (\text{BA}_{\text{intestinal phase}}/\text{BA}_{\text{initial phase}}) \times 100$$

where $\text{BA}_{\text{salivary phase}}$, $\text{BA}_{\text{gastric phase}}$, and $\text{BA}_{\text{intestinal phase}}$ corresponded to the area under the peaks at 330 nm in salivary, gastric, and intestinal phase, and $\text{BA}_{\text{initial}}$ to the area under the peak at 330 nm in the initial phase.

2.4. Determination of Neuroprotective Potential of Gingko Leaf Infusions

The acetylcholinesterase inhibition assay was carried out using a modified version of Ellman's reaction-based method [21]. A mixture was prepared by combining 25 μL of enzyme solution (0.25 U/mL), 125 μL of Ellman's reagent (3 mM), and 50 μL of ginkgo leaf infusion. This mixture was preincubated for 15 min at room temperature. Afterward, 25 μL of S-acetylthiocholine iodide (15 mM) was added, followed by an additional 15 min incubation at room temperature. Absorbance was measured at 405 nm using a plate reader

(Azure Biosystems, Dublin, CA, USA). A blank control was prepared with Tris-HCl buffer (50 mM, pH 8, 25 °C). The results were presented as a percentage of inhibition.

2.5. Determination of Anti-Hyperpigmentation Potential of Ginkgo Leaf Infusions

The tyrosinase inhibition assay was performed according to the method outlined by Jakimiuk et al. [22], with slight modifications. Ginkgo leaf infusions (80 µL) were first pre-incubated with 40 µL of tyrosinase solution (250 U/mL) at room temperature (25 °C) for 10 min. Afterward, 80 µL of 3,4-dihydroxy-L-phenylalanine (3 mM) was added, and the mixture was incubated for another 10 min at room temperature. Absorbance was measured at 492 nm using a plate reader. A blank control was prepared using phosphate-buffered saline (PBS) buffer (100 mM, pH 6.8, 25 °C). The results were expressed as a percentage of enzyme inhibition.

2.6. Determination of Anti-Diabetic Potential of Ginkgo Leaf Infusions

The α-glucosidase inhibition assay was performed following the method of Tiwari et al. [23], with slight modifications. Ginkgo leaf infusions (100 µL) were incubated with 50 µL of α-glucosidase (1 U/mL) for 10 min at 37 °C. Afterward, 50 µL of 5 mM 4-nitrophenyl α-D-glucopyranoside was added to the solution. Absorbance was read at 405 nm after a 5 min incubation using a plate reader. A blank control using PBS buffer (100 mM, pH 6.8, 25 °C) was prepared. The results were expressed as a percentage of enzyme inhibition.

2.7. Determination of DPPH Scavenging Activity

The DPPH scavenging activity was evaluated following the slightly modified procedure described by Brand-Williams et al. [24]. For the assay, 20 µL of each ginkgo leaf infusion was combined with 980 µL of a 0.094 mM methanolic DPPH solution. After 45 min of incubation, the absorbance was recorded at 515 nm, and the inhibition of DPPH radicals was expressed as a percentage.

2.8. Statistical Analysis

All experiments were conducted with a minimum of three replicates, and the data are presented as the mean ± standard deviation. Statistical analyses were carried out using PAST software (version 4.15) [25]. A one-way ANOVA was applied, followed by Tukey's HSD test for post hoc multiple mean comparisons. Differences were deemed statistically significant when $p < 0.05$.

3. Results

3.1. Polyphenolic Content

In infusions prepared from green spring leaves, yellow autumn leaves, and fallen leaves, we determine total polyphenol and total flavonoid content (Table 1).

Table 1. Total polyphenol and total flavonoid content in infusions prepared from different ginkgo leaf types. Data labeled with different letters differed significantly at $p < 0.05$.

Leaf Type	Total Polyphenol Content [µg GAE/mg dw]	Total Flavonoid Content [µg CA/mg dw]
Spring leaves	12.21 ± 2.75 ^{c}	4.09 ± 0.64 ^{c}
Autumn leaves	38.94 ± 1.46 ^{a}	12.70 ± 0.84 ^{a}
Fallen leaves	30.83 ± 0.47 ^{b}	9.98 ± 0.27 ^{b}

The total polyphenol content was 12.21 ± 2.75 µg GAE/mg dw, 38.94 ± 1.46 µg GAE/mg dw, and 30.83 ± 0.47 µg GAE/mg dw for spring, autumn, and fallen leaves, respectively, with significantly higher levels observed in yellow leaves compared to green leaves. A similar trend was seen for total flavonoid content, which measured 4.09 ± 0.64 µg CE/mg dw,

12.70 ± 0.84 µg CE/mg dw, and 9.98 ± 0.27 µg CE/mg dw, respectively, for spring, autumn, and fallen leaves.

3.2. In Vitro Flavonoid Bioaccessibility

In the infusions made from spring (green) and autumn (yellow) leaves, we determined the bioaccessibility of flavonoids (Figure 1).

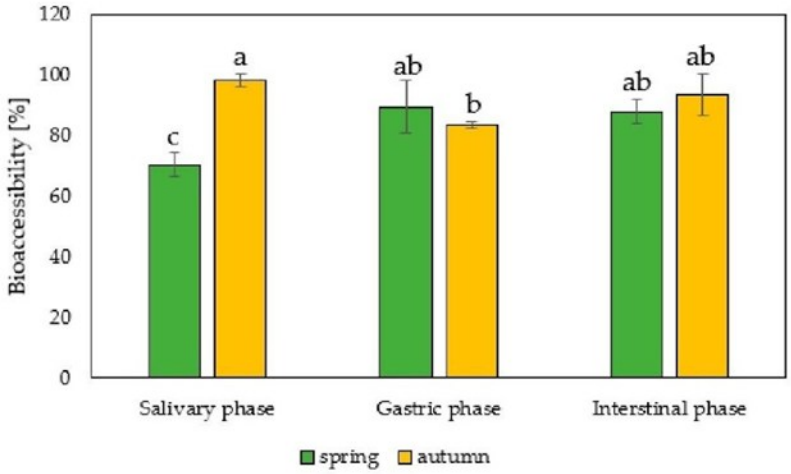

Figure 1. Bioaccessibility of flavonoids in ginkgo infusions prepared from green and yellow leaves. Data labeled with different letters differed significantly at $p < 0.05$.

In our HPLC analysis, no biflavonoids—typically the primary compounds in 70% ethanolic extracts—were detected. Based on the chromatograms and retention times (RT), the majority of flavonoids present in ginkgo leaves may be annotated as monomeric flavonoids or flavonoid glucosides. The total area under the curve for green leaves was 2870.18, while for yellow leaves, it was 4640.84, indicating 1.6 times more flavonoids in infusions from yellow leaves. For green leaf infusion, bioaccessibility in the salivary phase was lower (70.46 ± 4.06%) compared to the gastric (89.59 ± 8.74%) and intestinal phases (87.96 ± 4.15%). In yellow leaves, bioaccessibility was 98.23 ± 2.17% in the salivary phase, 83.42 ± 1.10% in the gastric phase, and 93.56 ± 6.91% in the intestinal phase.

3.3. In Vitro Neuroprotection Activity

In vitro neuroprotection activity was measured by observing the inhibition of acetylcholinesterase, and the results are shown at Figure 2.

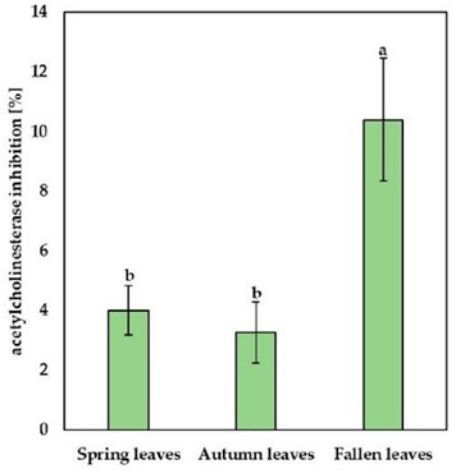

Figure 2. Inhibition of acetylcholinesterase (%) by infusions prepared from different ginkgo leaf types. Data labeled with different letters differed significantly at $p < 0.05$.

Spring and autumn leaves showed similar inhibition, around 3–4%, while fallen leaves showed higher inhibition potential against acetylcholinesterase, amounting to 10.40 ± 2.06%.

3.4. In Vitro Anti-Diabetic Potential

In vitro anti-diabetic potential was measured as α-glucosidase inhibition potential (Figure 3).

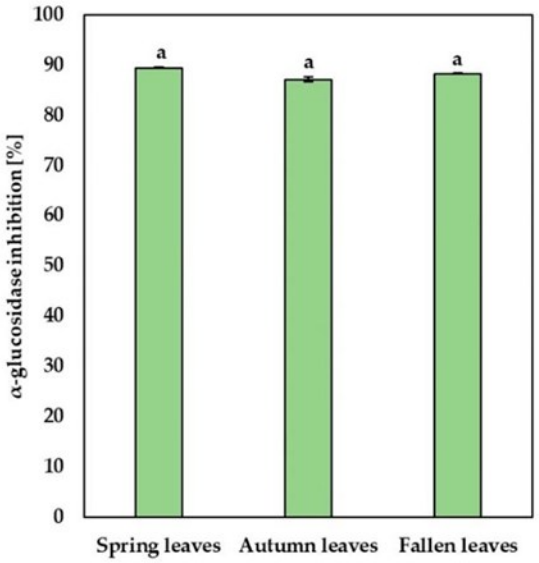

Figure 3. Inhibition of α-glucosidase (%) by infusions prepared from different ginkgo leaf types. Data labeled with different letters differed significantly at $p < 0.05$.

All three analyzed infusions showed α-glucosidase inhibition of around 90% and did not differ significantly.

3.5. Anti-Melanogenic Effect

Anti-melanogenic effect was measured as function of tyrosinase inhibition activity (Figure 4).

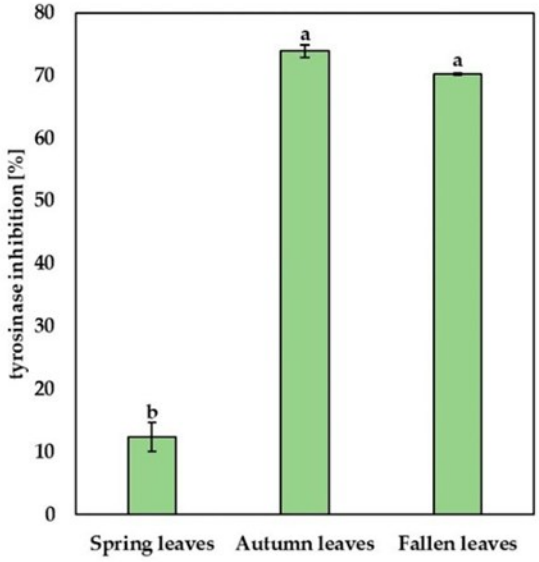

Figure 4. Inhibition of tyrosinase (%) by infusions prepared from different ginkgo leaf types. Data labeled with different letters differed significantly at $p < 0.05$.

Yellow leaves collected in autumn, as well as fallen leaves, exhibit significantly higher tyrosinase inhibition (73.89 ± 1.04% and 70.26 ± 0.12%, respectively) compared to the infusion made from green leaves (12.36 ± 2.29%).

3.6. Antioxidant Activity

Antioxidant activity of infusions was measured using DPPH radical and expressed as % of DPPH radical inhibition (Figure 5).

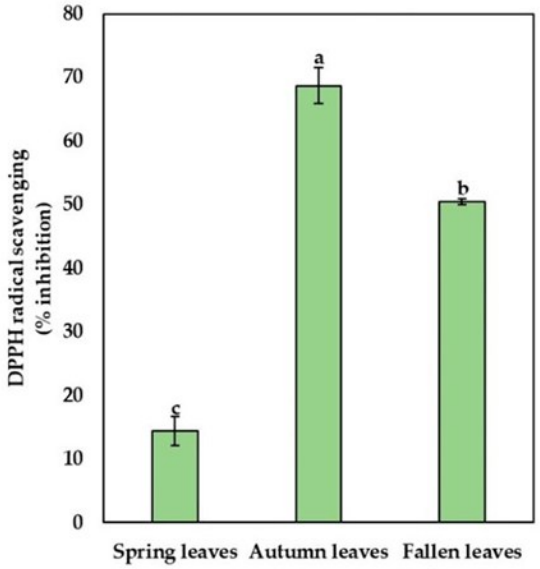

Figure 5. DPPH radical inhibition (%) by infusions prepared from different ginkgo leaf types. Data labeled with different letters differed significantly at $p < 0.05$.

Yellow leaves exhibit significantly higher antioxidant capacity compared to green leaves. Infusions prepared from yellow leaves collected directly from trees were more effective at inhibiting DPPH radicals than those made from fallen yellow leaves.

4. Discussion

Plant development stages significantly influence the accumulation of specialized metabolites, with different compounds often peaking at specific growth phases to support functions like defense, signaling, or reproduction [26]. Thus, preparations for potential medicinal use may vary in their properties depending on the plant's developmental stage, as the concentration and composition of bioactive compounds fluctuate throughout growth [26]. Herein, we compared ginkgo leaf infusions prepared from green leaves collected in spring and yellow leaves harvested in autumn, both directly from the tree and from fallen leaves. Collecting leaves after they have fallen, rather than harvesting them directly from the tree, can lower labor requirements and prevent damage to the tree, offering a more sustainable approach to leaf collection. The total polyphenol and flavonoid content followed a similar pattern, with significantly higher amounts found in both types of yellow leaves. A similar trend was reported by Kobus-Cisowska et al. [15], who compared the polyphenol and flavonoid content in PBS/ethyl acetate fractions of green and yellow ginkgo leaves, finding nearly double the content in yellow leaf extracts. Su et al. [27] also reported higher flavonoid content in yellow than in green leaves. This aligns with our results, where yellow leaves—both from trees and fallen—showed approximately 3.2-fold and 2.5-fold higher polyphenol and flavonoid content, respectively, compared to infusions made from green leaves. In total polyphenol and flavonoid content, we observed slightly higher amounts in yellow leaves collected directly from the trees, suggesting that some degradation of these compounds occurs in fallen leaves. Nevertheless, the levels in fallen leaves remained higher than those found in spring leaves. Although some published re-

ports [12] suggest that infusions contain fewer polyphenols compared to extracts prepared with organic solvents, our current results are consistent with previously published findings on the total flavonoid and polyphenolic content in ginkgo leaf extracts prepared with 80% methanol [28] and 70–80% ethanol [14,29,30]. Da Silva Pinto et al. [13] compared total polyphenol content in infusions and 12% ethanol extracts and reported higher content in infusions. In our study, HPLC–DAD analysis of the infusions did not detect biflavonoids, a group of dimeric flavonoids that were among the most abundant flavonoids identified in ginkgo leaf extracts prepared with 70% ethanol [14] and 80% methanol [19], as well as in those prepared using deep eutectic solvents [28]. This absence is likely due to their insolubility in water [31]. The flavonoids found in the infusions can be annotated as monomeric flavonoids and flavonoid glycosides (Supplementary Materials), with a higher abundance observed in yellow leaves, as confirmed by spectrophotometric methods. In the salivary phase for green leaves, we noted lower bioaccessibility compared to yellow leaves. However, after all three digestion phases, we did not observe a significant reduction during the intestinal digestion phase. Zhou et al. [32] evaluated the in vitro digestion of ginkgo leaf decoction and commercial capsules, noting that individual compounds underwent more significant changes during the intestinal and complete digestion stages, while antioxidant activity increased following in vitro digestion. Polyphenol and flavonoid content in plants often correlates well with antioxidant activity, as these compounds are known to possess strong antioxidant properties. Our results similarly show higher antioxidant activity in yellow leaves, both fallen and those collected directly from trees. This trend aligns with previous findings by Da Silva Pinto et al. [13], who reported higher DPPH inhibition capacity in infusions prepared from leaves collected in autumn. However, they also observed significant variations between trees, and in some instances, comparable antioxidant activity was found in both green and yellow leaves. Thus, concerning polyphenolic compounds in ginkgo, our results indicate that the developmental stage plays a significant role. Additionally, we must consider factors such as the age of the tree and variability between individual trees.

Fallen leaves and autumn leaves exhibit significantly higher inhibition of acetylcholinesterase compared to green leaves, indicating that leaves at later developmental stages contain more bioactive compounds with these activities. The inhibition of acetylcholinesterase is associated with neuroprotective potential, and ginkgo is widely recognized for its neuroprotective properties [33]. Compounds contributing to this activity include flavonoids, particularly the dimeric forms such as ginkgetin [34], which are abundant in yellow ginkgo leaves [20] and have been shown to strongly inhibit acetylcholinesterase [35]. Another study also supports the finding that yellow ginkgo leaves exhibit stronger cholinesterase inhibition activity compared to green leaves [15]. Different ginkgo extracts have been studied for acetylcholinesterase inhibition [36]; however, our report demonstrates for the first time that infusions also exhibit this activity, although likely at lower levels compared to other extracts. Notably, the highest inhibition recorded in fallen leaves was only 10.4%.

Our data showed the high inhibition activity of α-glucosidase by all three tested infusion types, and we did not record any differences between tested infusions. This inhibition activity is related with potential use of infusions in the treatment of diabetes, since α- glucosidase inhibitors may inhibit the absorption of carbohydrates from the small intestine [37]. Da Silva Pinto et al. [13] also examined the inhibition activity of α-glucosidase using ginkgo leaf infusions and ethanolic extracts. The results showed dose-dependent inhibition and variability between trees from which the leaves were collected. Consistent with our findings, the activity of infusions prepared from leaves harvested in both October and June was comparable. A recent docking and in vitro study by Li et al. [38] examined the α-glucosidase inhibitory activity of various compounds from ginkgo leaves, identifying quercitrin and quercetin as potential inhibitors. These compounds, being water-soluble, are present in ginkgo infusions [7,39,40].

Tyrosinases, enzymes responsible for melanin production across all life domains, are targeted by inhibitors used both to prevent certain skin disorders and as active ingredients in skin-whitening products [41]. In our study, significantly higher tyrosinase inhibition was observed in both types of yellow leaf infusions compared to green leaves, suggesting that yellow leaves could also be utilized in such preparations. Ginkgo leaf extracts prepared from leaves collected in September in China with 80% methanol also demonstrated tyrosinase inhibition activity [29]. Additionally, Ku et al. [42] showed that ethanolic extracts from green ginkgo leaves inhibited α-MSH-induced melanogenesis in murine melanoma (16F10) cells by downregulating melanogenic protein expression. Compounds in ginkgo associated with tyrosinase inhibition include flavonoid aglycones and terpene lactones [43]. However, ginkgo leaves also contain ginkgolic acids, which exhibit tyrosinase inhibition but can cause toxic or allergic reactions when ingested in high amounts; their content is therefore limited to 5 ppm in extracts by the European Pharmacopeia. Su et al. [27] compared green and yellow ginkgo leaf extracts, reporting significantly higher levels of ginkgolic acids in yellow leaves. This may suggest that our yellow leaf infusions have higher tyrosinase inhibition due to increased ginkgolic acid content, but it also raises concerns regarding the safety of using yellow leaf extracts for infusion preparations. Further toxicological studies are needed to clarify this potential risk.

5. Conclusions

We prepared infusions from ginkgo leaves collected from the tree in both spring and autumn, as well as from fallen autumn leaves. In the infusions made from yellow leaves (both collected from the tree and fallen), we found significantly higher total polyphenol and flavonoid content as well as good flavonoid bioaccessibility, along with greater tyrosinase inhibition and antioxidant activity. However, only the infusions from fallen leaves demonstrated superior activity, while α-glucosidase inhibition was comparable across all three infusion types. Our findings suggest that ginkgo leaf infusions, particularly those made from yellow leaves, could have promising applications in clinical, pharmaceutical, and cosmetic fields due to their neuroprotective, anti-hyperpigmentation, and anti-diabetic properties. Specifically, these infusions could be explored as natural ingredients in cosmetic formulations aimed at skin lightening. In clinical or pharmaceutical settings, they may be investigated further for supportive therapies in neurodegenerative diseases or blood sugar management. Future research should focus on detailed toxicological assessments to establish safety, as well as clinical studies to confirm efficacy in these applications. Additionally, potential seasonal variation should be considered, as the current research presents data from only one season. Studies on optimizing extraction methods and assessing bioavailability in human models would further elucidate the therapeutic potential of ginkgo leaf infusions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app142210231/s1.

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5.2.3. From Waste to Resource: Valorization of Yellow Ginkgo Leaves as a Source of Pharmacologically Relevant Biflavonoids

applied sciences

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From Waste to Resource: Valorization of Yellow Ginkgo Leaves as a Source of Pharmacologically Relevant Biflavonoids

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Featured Application

Fallen yellow ginkgo leaves, typically treated as waste in urban and landscaped areas, can be valorized as a sustainable and non-invasive source of pharmacologically relevant biflavonoids. Their high content of sciadopitysin, ginkgetin, isoginkgetin, and related compounds makes them suitable for development into standardized extracts or purified fractions with potential applications in pharmaceuticals, nutraceuticals, and functional foods. Moreover, the utilization of naturally shed leaves reduces environmental waste while avoiding disruption of the ginkgo tree's physiological cycle, offering a scalable and eco-friendly raw material supply for bioactive product development.

Abstract

Ginko (*Ginkgo biloba* L.) is a widely distributed ornamental tree that produces large quantities of leaves annually, turning golden yellow in autumn due to chlorophyll degradation and carotenoid retention. While green ginkgo leaves and standardized extracts have been extensively studied, senescent and naturally fallen leaves remain only scarcely investigated, despite representing a substantial biomass resource. In this study, we analyzed yellow ginkgo leaves collected directly from trees and those naturally shed at four time points during autumn. We determined pigment composition, total polyphenols, flavonoids, phenolic acids, and the concentrations of five major biflavonoids. Chlorophylls decreased progressively in tree-collected leaves, whereas carotenoid levels remained stable or slightly elevated. Polyphenolic compounds were more abundant in fallen leaves. Biflavonoid profiling revealed the presence of amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin, with sciadopitysin as the most abundant. Total biflavonoid content reached up to 8 mg/g dw, with higher levels in fallen leaves compared to those collected from the tree. These findings highlight yellow ginkgo leaves, particularly fallen ones, as a sustainable and non-invasive source of pharmacologically relevant biflavonoids. However, further research is needed to optimize eco-friendly extraction strategies and to evaluate safety aspects.

Keywords: ginkgo; biflavonoids; yellow leaves; sustainable resource utilization

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1. Introduction

Flavonoids are one of the most widespread and structurally diverse classes of plant polyphenols, fulfilling essential roles in pigmentation, UV protection, defense, and signaling. Their structural core, derived from the phenylpropanoid pathway, allows for numerous chemical modifications that expand diversity and modulate function. Beyond

monomeric forms, flavonoids also undergo dimerization, producing a unique group of compounds collectively referred to as biflavonoids. According to the review article by He et al. [1], more than 592 biflavonoids have been structurally elucidated and may show a wide range of pharmacological activities, including anti-inflammatory, antioxidant, antibacterial, antiviral, antidiabetic, antitumor, and cytotoxic properties. Moreover, biflavonoids have demonstrated promising activity in experimental and preclinical models related to neurodegenerative diseases such as Alzheimer's and Parkinson's [2–4]. The dimerization of flavonoids significantly extends their functional landscape. For example, biflavonoids are better acetylcholinesterase, α-amylase and α-glucosidase inhibitors than their monomeric subunits, which indicates their potential role in the prevention of neurodegenerative diseases and diabetes [5].

Unlike monomeric flavonoids, which are widely distributed across the plant kingdom, biflavonoids are comparatively rare and not universally present in all plant species [1]. Their exact distribution remains incompletely characterized, as systematic surveys across taxa are still limited. Current evidence suggests that biflavonoids occur sporadically in certain lineages, often with taxonomic or ecological significance. Some examples of biflavonoid sources include *Selaginella* (Lycophytes) [6], *Hypericum* (Clusiaceae) [7], and *Juniperus* (Cupressaceae) [8], where they contribute to structural diversity, antimicrobial activity, and chemical defense.

Biflavonoids were first isolated from yellow ginkgo (*Ginkgo biloba* L.) leaves, which remains one of the best-known and most extensively studied sources; in this species, characteristic 3′,8″-linked biflavones such as amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin have been identified [9]. The standardized *G. biloba* preparation, EGb761, contains 24% ginkgo flavonoids and 6% terpene trilactones (TTLs) and has long been used in the treatment of asthma, bronchitis, ischemia, arteriosclerosis, and rheumatism [10]. Yellow ginkgo leaf extracts may exhibit stronger biological activity than those from green leaves [11–15]. Our recent report shows higher accumulation of biflavonoids in yellow leaves [16], and yellow ginkgo leaves contain higher amounts of terpene trilactones bilobalide and ginkgolides B and C [17]. Therefore, senescent yellow ginkgo leaves may represent a valuable source of biflavonoids with potential pharmaceutical applications.

Given the large size of many ginkgo trees and their wide distribution across the globe in urban parks, streetscapes, and landscaped areas, the annual leaf fall represents a substantial amount of biomass. At present, these fallen ginkgo leaves are generally managed as green waste and are removed and discarded without further utilization. Although there have been reports suggesting alternative applications, for example, the use of ginkgo leaf biomass in biochar production [18], the vast majority of leaves remain unexploited and are ultimately treated as waste.

In this study, we investigated the potential of yellow ginkgo leaves, both those collected directly from the tree and those naturally senesced and fallen, as a source of valuable phytochemicals, with particular emphasis on biflavonoids. While ginkgo leaves are well known to contain a wide range of metabolites with pharmacological significance [19], to the best of our knowledge, no previous studies have specifically addressed the presence, concentration, or stability of biflavonoids in senescent or fallen leaves. Assessing this overlooked biomass could open new opportunities for sustainable resource utilization, help reduce organic waste in urban environments, and support the development of novel bioactive compounds from a currently neglected natural source.

To this end, we analyzed seasonal changes in biflavonoid content in yellow ginkgo leaves by determining pigment concentration, total polyphenols, flavonoids, phenolic acids, and the concentrations of five major biflavonoids—amentoflavone, bilobetin, ginkgetin, isoginkgetin and sciadopitysin. Both tree-collected and naturally fallen leaves were ex-

amined at four different time points during autumn, providing insight into how leaf senescence and natural abscission influence the accumulation and preservation of these pharmacologically relevant compounds.

2. Materials and Methods

2.1. Chemicals and Instruments

For this research, the following equipment and reagents were employed. Ginkgo leaves were freeze-dried using a LIO-5PLT (KAMBIČ, Ljubljana, Slovenia), and the resulting material was ground to a fine powder with a Bead Ruptor 12 (Omni International, Kennesaw, GA, USA). Plant material was weighed on an analytical balance (Adam Equipment, Maidstone, United Kingdom). Extractions were carried out with 70% ethanol (GRAM-MOL, Zagreb, Croatia) employing an ultrasonic bath (DU-100, Argo Lab, Carpi, Italy), followed by mixing on a mechanical rotator (Bio RS-24, Biosan, Riga, Latvia) and a vortex mixer (V-1 plus, Biosan, Riga, Latvia). The extracts were then clarified by centrifugation (LMC-4200R, Biosan, Riga, Latvia). For the determination of chlorophylls and carotenoids, acetone was used as the extraction solvent (GRAM-MOL, Zagreb, Croatia). UV-VIS measurements were performed on a spectrophotometer (ONDA TOUCH UV-21, China). For the determination of polyphenolic compounds, the following chemicals were used: gallic acid 98% (Acros Organics, Shanghai, China), caffeic acid $\geq$ 98% HPLC grade (Sigma Aldrich, St. Louis, Missouri, MO, USA), (+)-catechin hydrate (Sigma Aldrich, St. Louis, Missouri, MO, USA), Folin–Ciocalteu's reagent (Sigma Aldrich, Buchs, Switzerland), sodium carbonate (T.T.T., Sveta Nedelja, Croatia), aluminum chloride hexahydrate (Thermo Fischer Scientific, Kandel, Germany), hydrochloric acid 36.5% (Kemika, Zagreb, Croatia), sodium hydroxide (T.T.T., Sveta Nedelja, Croatia), sodium nitrite (Kemika, Zagreb, Croatia), sodium molybdate (VI) dihydrate (Sigma Aldrich, St. Louis, Missouri, MO, USA), and methanol (Kemika, Zagreb, Croatia). Biflavonoid quantification was carried out on an Agilent 1260 Infinity II high-performance liquid chromatography system (Agilent, Santa Clara, CA, USA) equipped with a diode array detector (DAD). The mobile phase consisted of acetonitrile ($\geq$99.9% UHPLC gradient grade; Fischer Scientific, Taipei City, Taiwan), ultrapure water, and formic acid (98–100%; Sigma Aldrich, Darmstadt, Germany). HPLC-grade standards of amentoflavone, ginkgetin, isoginkgetin, bilobetin, and sciadopitysin (PhytoLab, Vestenbergsgreuth, Germany) were prepared as stock solutions in pure dimethyl sulfoxide (Fisher Scientific, Loughborough, UK).

2.2. Leaves Collection and Storage

Ginkgo autumn leaves were collected in 2022; leaves from trees as well as fallen leaves were collected at four different time points (Table 1).

Table 1. Date of sampling of leaves from tree and fallen leaves.

	Leaves from Trees	Fallen Leaves
I.	3 October 2022	24 October 2022
II.	18 October 2022	2 November 2022
III.	2 November 2022	7 November 2022
IV.	7 November 2022	21 November 2022

Leaf material was collected from an alley of ginkgo trees (46°09′20″ N; 16°49′46″ E) located in the city of Koprivnica, Croatia. Sampling was carried out on five ginkgo trees of the same age (approximately 30 years), which nevertheless differed in the onset and intensity of leaf yellowing and abscission (Figure 1). In this study, some trees completed leaf

fall by mid-October, while in others the final leaf drop occurred as late as late November, so this is the reason why we collect samples at different time points, since the complete leaf drop occurs in one tree in less than 24 h.

Figure 1. Different dynamics of ginkgo leaf yellowing in late October.

Immediately after collection, leaf samples were stored at –80 °C until lyophilization. Freeze-drying was carried out for 48 h at approximately –102 °C under a pressure of 0.3303 mBar. The lyophilized material was then ground into a fine powder using a bead mill, which served as the starting material for subsequent extractions.

2.3. Determination of Pigment Content

For the quantification of chlorophylls and carotenoids in autumn ginkgo leaves, the method of Lichtenthaler and Buschmann [20] was applied. Approximately ten milligrams of finely ground leaf powder was weighed and extracted with 1 mL of pure acetone. The extracts were centrifuged, and the resulting supernatant was collected for analysis. Absorbance was measured at three wavelengths: 661.6 nm, 644.8 nm, and 470 nm. Pigment concentrations were calculated using the equations specific for pure acetone and results are expressed as micrograms per gram of dry weight (µg/g dw) of lyophilized leaf powder.

2.4. Determination of Polyphenolic Compounds

The determination of phenolic compounds included the measurement of total polyphenols, total flavonoids, total phenolic acids, and five major 3′,8″-biflavones from autumn ginkgo leaf extracts. Extract preparation was performed as follows: 60 mg of finely ground leaf powder was weighed for each sample and extracted with 2 mL of 70% ethanol. The extraction solvent and isolation protocol were selected based on our previously published work [16]. The mixture was vortexed briefly and then placed in an ultrasonic bath for 10 min at room temperature. Subsequently, the samples were mixed on a rotary mixer for 45 min and centrifuged at 4000 rpm for 10 min at room temperature. The resulting supernatant was collected and used for further analyses.

2.4.1. Total Polyphenols

Total polyphenol content was assessed using a modified Folin–Ciocalteu method [21]. In brief, 200 µL of ginkgo leaf extract was combined with 1580 µL of distilled water and 100 µL of Folin–Ciocalteu reagent, followed by the addition of 300 µL of saturated sodium carbonate solution. The mixture was incubated at room temperature for 2 h, and absorbance was then recorded at 765 nm. Quantification was performed against a calibration curve ($y = 0.0011x$, $R^2 = 0.99$) prepared with gallic acid in a range 0–2000 mg/L, and results

are expressed as gallic acid equivalents per milligram of dry weight (μg GAE/mg dw) of lyophilized leaf powder.

2.4.2. Total Flavonoids

The total flavonoid content was determined following the method described by Zhishen et al. [22]. In short, 200 μL of extract was combined with 800 μL of distilled water, after which 60 μL of 5% sodium nitrite ($NaNO_2$) solution was added to initiate the reaction. Following a 5 min incubation, 60 μL of 10% aluminum chloride (Al_2Cl_3) solution was introduced, and after an additional 6 min, 400 μL of 1 M sodium hydroxide (NaOH) was added. The absorbance was then measured at 510 nm. Quantification was performed using a catechin (0–500 mg/L) calibration curve ($y = 0.0023x$, $R^2 = 0.99$), and results are reported as catechin equivalents per milligram of dry weight (μg CE/mg dw) of lyophilized leaf powder.

2.4.3. Total Phenolic Acids

The total phenolic acid content was assessed using the Arnow reagent method [23]. Briefly, 300 μL of extract was combined with 300 μL of distilled water, 100 μL of 0.5 M hydrochloric acid (HCl), and 100 μL of Arnow's reagent (sodium nitrite and sodium molybdate dihydrate). This was followed by the addition of 100 μL of 1 M sodium hydroxide (NaOH) and 100 μL of distilled water. Absorbance was then recorded at 505 nm. Quantification was carried out using a calibration curve ($y = 0.0074x$, $R^2 = 0.98$) constructed with caffeic acid (0–250 mg/L), and results are expressed as caffeic acid equivalents per milligram of dry weight (μg CAE/mg dw) of lyophilized leaf powder.

2.4.4. Biflavonoid Profiling

The major 3′,8″-biflavones (amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin) in ginkgo leaf extracts were determined following the method of Jurčević Šangut and Šamec [16]. Detection and quantification were performed using an Agilent 1260 Infinity II high-performance liquid chromatography (HPLC) system (Agilent, Santa Clara, CA, USA) equipped with a diode array detector (DAD). Both samples and standards were injected at a volume of 10 μL. Prior to injection, ginkgo extracts were filtered through polytetrafluoroethylene (PTFE) syringe filters with a pore size of 0.45 μm.

Standard stock solutions (1 mg/mL) were prepared in pure DMSO, and serial dilutions with pure methanol yielded final concentrations of 1, 10, 50, and 100 μg/mL. Chromatographic detection was carried out at 330 nm, and data acquisition and processing were performed using Agilent OpenLab CDS software (version 2.6). Identification of the five biflavones was based on the comparison of UV–Vis spectra and retention times between sample peaks and their respective standards. Quantification was performed using calibration curves, and concentrations are expressed as micrograms per milligram of dry weight (μg/g dw) of lyophilized leaf powder. Chromatographic separation was performed using a Zorbax 300Extend-C18 column (150 × 4.6 mm, 3.5 μm; Agilent, Santa Clara, CA, USA) maintained at 40 °C. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B), with a multistep linear gradient program applied for analyte elution at a constant flow rate of 1.0 mL/min over a 45 min run. The gradient elution profile was as follows: 0 min 98% A, 10 min 79% A, 15 min 77% A, 20 min 75% A, 25 min 64% A, 30 min 62% A, 35 min 51% A, 40 min 25% A, 43 min 8% A, and 45 min 98% A.

2.5. Statistical Analysis

Leaf sampling was performed on five ginkgo trees, each serving as an independent biological replicate. All measurements were conducted in at least three technical replicates, and results are presented as mean ± standard deviation. Statistical analyses were carried

out using PAST software (version 4.15) [24], applying one-way ANOVA followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$, with values followed by different letters indicating statistically significant differences.

3. Results

3.1. Pigment Content

The content of green pigments, chlorophyll *a*, chlorophyll *b* and total chlorophylls as well as total carotenoids is shown in Table 2.

Table 2. The contents of chlorophyll *a*, chlorophyll *b*, total chlorophylls and total carotenoids in yellow leaves collected from trees and fallen leaves at four time points. Values are presented as mean ± SD and expressed as μg/g dw.

Leaves from Trees				
	Chl *a* (μg/g dw)	Chl *b* (μg/g dw)	Total Chls (μg/g dw)	Total Car (μg/g dw)
I	611.87 ± 19.43 ^{a}	105.42 ± 8.75 ^{a}	717.29 ± 28.03 ^{a}	66.70 ± 4.63 ^{d}
II	232.96 ± 9.03 ^{b}	52.67 ± 6.72 ^{a}	285.62 ± 11.19 ^{b}	63.77 ± 7.79 ^{d}
III	98.95 ± 12.15 ^{cd}	87.92 ± 20.70 ^{a}	186.87 ± 32.84 ^{cd}	90.95 ± 0.78 ^{c}
IV	126.09 ± 4.41 ^{c}	91.94 ± 5.37 ^{a}	218.03 ± 9.37 ^{bc}	105.48 ± 1.38 ^{b}
Fallen leaves				
	Chl *a* (μg/g dw)	Chl *b* (μg/g dw)	Total Chls (μg/g dw)	Total Car (μg/g dw)
I	57.25 ± 7.57 ^{de}	63.20 ± 10.14 ^{a}	120.44 ± 17.70 ^{d}	110.61 ± 1.56 ^{b}
II	56.63 ± 10.18 ^{e}	87.22 ± 16.45 ^{a}	143.85 ± 26.54 ^{cd}	143.47 ± 2.08 ^{a}
III	63.88 ± 13.41 ^{de}	93.49 ± 29.70 ^{a}	157.37 ± 43.00 ^{cd}	73.27 ± 0.85 ^{d}
IV	68.86 ± 14.28 ^{de}	69.61 ± 20.97 ^{a}	138.47 ± 35.26 ^{cd}	153.26 ± 1.08 ^{a}

Statistical significance was determined at $p < 0.05$, and values marked with different letters denote statistically significant differences.

As expected, the contents of chlorophyll *a*, chlorophyll *b*, and total chlorophyll gradually decreased in leaves collected from trees from the beginning of sampling (I) to the end of the growing season (IV), reflecting the typical physiological response to senescence. In fallen leaves, although no longer photosynthetically active, chlorophylls were still detected; their levels remained relatively stable across the different collection time points but were significantly lower than in leaves collected directly from the trees. In leaves from the tree, carotenoid content increased with time, and in fallen leaves at three time points, the content was higher than in leaves from the tree.

3.2. Polyphenolic Compounds Content

3.2.1. Total Polyphenols, Flavonoids and Phenolic Acids

The content of total polyphenols (TP), total flavonoids (TF) and total phenolic acids (TPA) in analyzed yellow ginkgo leaves is shown in Table 3.

In general, the total contents of polyphenols, flavonoids, and phenolic acids were higher in fallen leaves compared to those collected directly from the tree, although the collection date had a significant influence. The highest total polyphenol and flavonoid content was detected in leaves from the tree sampled at the second sampling date (II). The highest polyphenol, flavonoid and phenolic acid content was observed in fallen leaves collected at the first sampling date (I).

Table 3. The content of polyphenols (TP), total flavonoids (TF) and total phenolic acids (TPA) in yellow ginkgo leaves collected from the tree and fallen leaves.

	Leaves from Trees		
	TP (μg GAE/mg dw)	TF (μg CE/mg dw)	TPA (μg CAE/mg dw)
I	33.52 ± 0.50 ^{g}	4.10 ± 0.26 ^{d}	0.95 ± 0.01 ^{c}
II	45.91 ± 0.32 ^{d}	5.26 ± 0.17 ^{c}	1.00 ± 0.04 ^{c}
III	41.85 ± 0.40 ^{e}	5.01 ± 0.17 ^{cd}	1.07 ± 0.13 ^{bc}
IV	38.60 ± 0.23 ^{e}	4.74 ± 0.10 ^{cd}	1.19 ± 0.04 ^{bc}
	Fallen leaves		
	TP (μg GAE/mg dw)	TF (μg CE/mg dw)	TPA (μg CAE/mg dw)
I	62.52 ± 2.41 ^{a}	62.52 ± 2.41 ^{a}	62.52 ± 2.41 ^{a}
II	58.80 ± 1.01 ^{b}	58.80 ± 1.01 ^{b}	58.80 ± 1.01 ^{b}
III	38.28 ± 0.16 ^{f}	38.28 ± 0.16 ^{f}	38.28 ± 0.16 ^{f}
IV	49.49 ± 0.81 ^{c}	49.49 ± 0.81 ^{c}	49.49 ± 0.81 ^{c}

Statistical significance was determined at $p < 0.05$, and values marked with different letters denote statistically significant differences.

3.2.2. Biflavonoids

In all collected samples of yellow ginkgo leaves, we especially focused on the determination of five major biflavonoids, amentoflavone, bilobetin, ginkgetin, isoginkgetin and sciadopitysin, using HPLC-DAD. Representative chromatograms with all five biflavonoids peaks are shown in Figure 2.

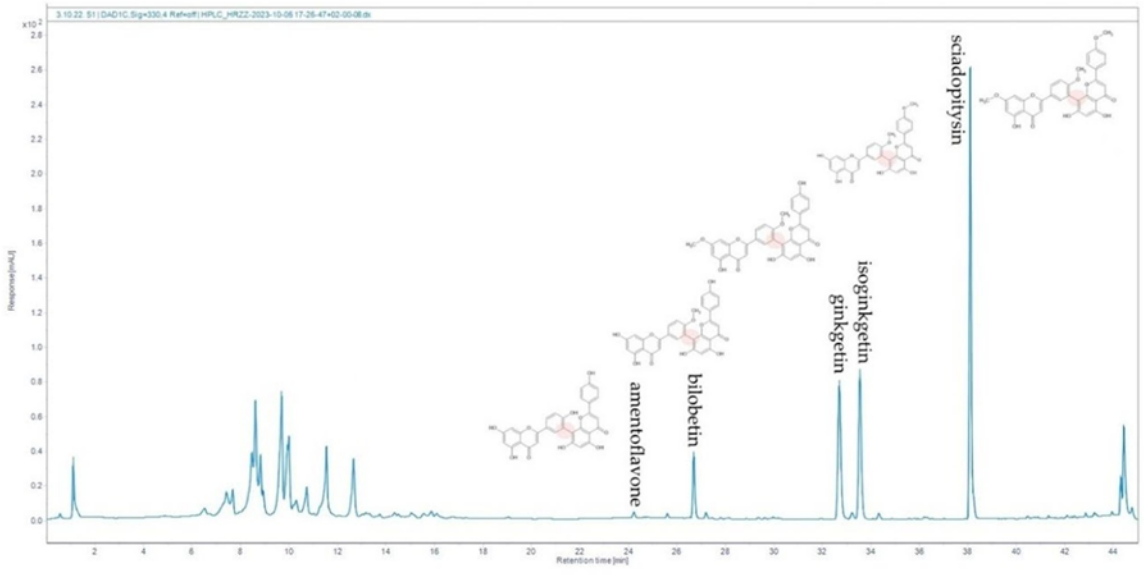

Figure 2. Yellow ginkgo leaf extract chromatogram recorded at 330 nm.

All five biflavonoids were detected in every sample, although their levels varied depending on the collection date and leaf type. The results are presented in Table 4.

Table 4. The content of individual biflavonoids (μg/g dw), amentoflavone, bilobetin, ginkgetin, isoginkgetin and sciadopitysin, in yellow ginkgo leaves from trees and fallen leaves.

Leaves from Trees				
	I	II	III	IV
Amentoflavone	48.82 ± 14.22 ^{b}	49.94 ± 13.25 ^{b}	57.69 ± 22.85 ^{bd}	55.43 ± 17.67 ^{b}
Bilobetin	469.77 ± 124.24 ^{b}	494.59 ± 151.56 ^{b}	526.82 ± 220.06 ^{b}	519.16 ± 179.52 ^{b}
Ginkgetin	1022.66 ± 177.51 ^{b}	1133.47 ± 235.31 ^{b}	1091.05 ± 452.31 ^{b}	1170.72 ± 229.13 ^{b}
Isoginkgetin	1172.25 ± 280.80 ^{bd}	1234.57 ± 340.23 ^{bcd}	1137.08 ± 697.23 ^{b}	1278.48 ± 392.13 ^{a}
Sciadopitysin	2060.55 ± 278.71 ^{b}	2242.87 ± 360.21 ^{bc}	2394.96 ± 465.57 ^{a}	2346.62 ± 392.29 ^{bc}
Fallen leaves				
	I	II	III	IV
Amentoflavone	88.20 ± 1.86 ^{acd}	102.20 ± 16.38 ^{a}	67.11 ± 0.47 ^{bc}	92.29 ± 1.07 ^{ac}
Bilobetin	870.99 ± 19.99 ^{a}	915.22 ± 28.89 ^{a}	656.23 ± 3.39 ^{b}	851.95 ± 17.32 ^{a}
Ginkgetin	1675.26 ± 35.86 ^{a}	1497.30 ± 57.26 ^{ab}	1350.85 ± 11.13 ^{ab}	1324.33 ± 20.35 ^{ab}
Isoginkgetin	2006.11 ± 42.25 ^{a}	1962.37 ± 26.58 ^{ac}	1599.52 ± 11.69 ^{a}	1896.62 ± 39.60 ^{ad}
Sciadopitysin	2994.26 ± 60.67 ^{a}	2738.20 ± 32.41 ^{ac}	2685.81 ± 23.45 ^{ac}	2707.69 ± 42.57 ^{ac}

Statistical significance was determined at $p < 0.05$, and values marked with different letters denote statistically significant differences.

In all analyzed samples, the most abundant biflavonoid was sciadopitysin, followed by isoginkgetin and ginkgetin. Bilobetin and amentoflavone were consistently present at concentrations below 1000 μg/g dw. All fallen leaf samples showed a higher amount of biflavonoids, which is also evident from the content of total biflavonoids presented in Figure 3.

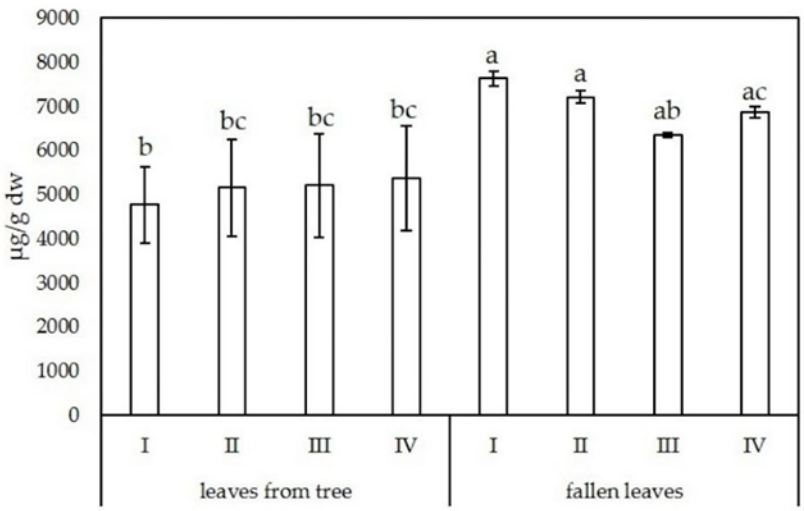

Figure 3. Total biflavonoid content in yellow ginkgo leaves collected from trees and fallen leaves. Statistical significance was determined at $p < 0.05$, and values marked with different letters denote statistically significant differences.

The total biflavonoid content ranged from 4774.05 to 5370.40 μg/g dw in leaves collected from the tree and from 6359.52 to 7634.82 μg/g dw in fallen leaves, indicating that fallen leaves contain higher levels of biflavonoids.

4. Discussion

Ginkgo is a well-known ornamental plant that turns golden yellow in autumn. This color change results from the loss of green pigments (chlorophylls) [25–27], as is evident also from our results in Table 2 by their gradual decrease over time in leaves col-

lected from the trees. According to Tang et al. [27], chlorophyll degradation in ginkgo involves enzyme chlorophyllase, whose activity is highest in green leaves and significantly declines during the process of leaf yellowing. Since fallen leaves are no longer photosynthetically active, their chlorophyll content is, as expected, very low (Table 2). In contrast, the carotenoid content in our samples is stable and even higher in fallen leaves. Zhang et al. [28], Yang et al. [25] and Li et al. [29] reported comparable amount of carotenoids in ginkgo leaves collected during October and November. Several studies have reported that the yellow coloration of ginkgo leaves is associated with their carotenoid levels [28,30], but exact mechanisms are still under investigation, with particular attention in recent years to combining transcriptomic and metabolomics data [26,29–31]. Carotenoids represent a diverse group of biologically active phytochemicals, and their potential health effects are mainly attributed to pronounced antioxidant and singlet oxygen-quenching activities [32]. Epidemiological studies consistently indicate that higher dietary intake and elevated tissue concentrations of carotenoids are associated with a lower incidence of various chronic disorders, including cardiovascular disease, type 2 diabetes mellitus, obesity, neurodegenerative conditions, and several forms of cancer [32].

According to our findings, yellow ginkgo leaves represent a significant source of polyphenolic compounds (Tables 3 and 4, Figure 3). The total concentrations of polyphenols, flavonoids, and phenolic acids were higher in fallen leaves than in those collected directly from the trees (Table 3). This pattern is likely related to the degradation of complex polyphenolic structures after leaf abscission, which leads to the release of simpler monomeric compounds that can be more readily detected using the applied analytical methods. Postharvest degradation of plant cell walls results from the activity of cell wall-degrading enzymes, leading to the depolymerization of polyphenolic compounds. Consequently, this may manifest as an apparent increase in total polyphenolic content, a phenomenon also documented in previous studies [33]. Polyphenolic compounds constitute one of the principal biologically active components of ginkgo extracts [34,35]. Liu et al. [19] summarized information about ginkgo biologically active compounds published from 2015 to 2020 and found that in ginkgo 110 flavonoids were reported with unambiguous structures. These compounds include 52 flavonol glycosides, seven flavonols, 14 flavone glycosides and five flavones, two flavanones and one flavanone glycoside, two isoflavones and one isoflavone glycoside, four flavan-3-ols, 13 biflavonoids, and nine biginkgosides. In recent years, biflavonoids, naturally occurring flavonoid dimers, have gained increasing attention due to their diverse pharmacological potential [13,36–39]. In our study, we quantified biflavonoids in yellow ginkgo leaves, comparing samples collected directly from trees with those naturally fallen to the ground (Table 4, Figure 3). Across all samples, we identified amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin. This profile is consistent with previous reports, which describe these compounds as the predominant biflavonoids in ginkgo leaves [9,40,41]. Among the identified biflavonoids, sciadopitysin was the most abundant in all our analysed samples, in agreement with some literature data [42,43]. However, other studies have reported isoginkgetin [44] or ginkgetin [45] as the dominant biflavonoid, suggesting that environmental conditions, plant age, or seasonal factors may influence biflavonoid composition.

Our quantitative analysis revealed that the total biflavonoid content reached up to 8 mg/g dry weight (dw) in yellow leaves (Figure 3), confirming that ginkgo leaves are a valuable natural source of these compounds. Previous studies also showed that yellow gingko leaves are more abundant in biflavonoids than green ones [16,40]. Interestingly, we observed even higher concentrations of biflavonoids in fallen leaves compared to leaves still attached to the tree (Table 4, Figure 3). This finding highlights the potential of utilizing naturally shed leaves as a sustainable and non-invasive source of biflavonoids. By harvest-

ing fallen leaves, it may be possible to obtain substantial quantities of pharmacologically relevant compounds without disrupting the normal physiological cycle of the ginkgo tree. However, further studies are needed to optimize extraction protocols for the efficient isolation of biflavonoids from fallen ginkgo leaves. Previously, Shen et al. [46] reported a protocol for large-scale, targeted isolation of biflavonoids with high purity from industrial ginkgo exocarp using two-dimensional chromatography, although their methodology was not specifically applied to leaf-derived biflavonoids. In our earlier research, we explored the potential of green extraction techniques [47], including deep eutectic solvents (DESs) [48], as sustainable approaches for biflavonoid isolation. While these methods show promise, additional optimization is required to improve yield, selectivity, and scalability. At the same time, there are some concerns regarding the direct use of yellow ginkgo leaves in traditional preparations such as infusions or wine due to the presence of potentially undesirable constituents [49]. For example, Horbowicz et al. [17] reported that the concentrations of major terpene trilactones ginkgolides B and C and bilobalide were significantly higher (higher than recommended values) in the leaf blades of naturally senesced yellow leaves compared with green leaves. Therefore, further studies are necessary to assess the safety, efficacy, and suitability of such applications, while also developing reliable purification strategies to ensure that ginkgo-derived products meet both pharmacological and safety standards.

5. Conclusions

Our study demonstrates that yellow ginkgo leaves, both collected from the tree and naturally senesced, represent a valuable source of biologically active phytochemicals. Pigment analysis confirmed chlorophyll degradation and carotenoid persistence as key features of leaf yellowing, consistent with previous reports. Polyphenolic and biflavonoid contents were higher in fallen leaves, with sciadopitysin identified as the most abundant biflavonoid across all samples. The total biflavonoid concentration, reaching up to 8 mg/g dw, positions ginkgo leaves among significant natural sources of these pharmacologically important compounds.

Importantly, the higher levels detected in naturally fallen leaves suggest a sustainable opportunity for their use in biflavonoid production without interfering with the physiological cycle of the tree or generating unnecessary biomass waste. This finding supports the concept of valorizing ginkgo leaf litter as an underutilized resource in urban and landscape environments. Nevertheless, further studies are required to: (i) optimize extraction and purification protocols, particularly using green technologies; (ii) clarify variability in biflavonoid composition due to environmental or seasonal factors; and (iii) rigorously assess the safety of preparations from yellow leaves, with particular attention to potential risks such as microbial contamination, mycotoxins, chemical degradation, and variability in quality. Addressing these aspects will be critical for the safe and efficient utilization of fallen ginkgo leaves as a renewable source of bioactive compounds for pharmaceutical, nutraceutical, and functional applications.

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Curriculum vitae

Iva Jurčević Šangut was born on October 25, 1997, in Zagreb. After completing high school education at the 11th Gymnasium in 2016, she enrolled in the undergraduate study programme in Biology at the Department of Biology, Josip Juraj Strossmayer University of Osijek. In the autumn of 2021, she graduated from the research track of the same Department. During her studies, she actively participated in science popularization, served as a teaching demonstrator in laboratory courses, and engaged in scientific research focused on hematophagous vectors important in the context of public health. She presented the results of her research at several international conferences and received an award for one of the best poster presentations at the EMCA conference. Alongside her studies, she was actively involved in the work of the association Duhos, dedicating particular attention to youth and people in need in the city of Osijek. Since July 2022, she has been employed as a research assistant on the project "The Role of Biflavonoids in Plants - *Ginkgo biloba* L. as a Model System" (UIP-2019-04-1018), carried out at the University North, Department of Food Technology in Koprivnica, under the supervision of Assistant Professor Šamec, PhD. In the same year, she enrolled in the Doctoral Programme in Biology at the Faculty of Science, University of Zagreb, Department of Biology. In addition to her main project, she participates as a collaborator in several institutional projects and one ERASMUS+ project, and is highly active in science popularization activities. Beyond research work, she is also involved in teaching several courses at the University North, including Fundamentals of Biology, Food Microbiology, and Fundamentals of Biochemistry, where she fosters a special interest and enthusiasm for working with students. By February 2026, she has coauthored 13 original scientific papers, predominantly published in Q1/Q2 journals, coauthored 2 book chapters, and participated in 21 international conferences. According to Google Scholar report her articles are cited 119 times, and her *h*-index is 6. She has received an award for best poster presentation at the international conference Food and Climate Change conference in 2023 and was a grantee of the Federation of European Societies of Plant Biology for participation in the Plant Biology Europe conference in Budapest in 2025. She is a proud member of the BiolOS Alumni Association, the Croatian Society of Plant Biology, and the Croatian Biological Society.

Scientific contribution and activities

CroRIS ID: 42475

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13. **Jurčević Šangut, Iva**; Oreški, Rebeka; Šamec, Dunja. Comparison of the Biflavonoid Profile of Ginkgo (*Ginkgo biloba* L.) Leaves Extracts Prepared with Different Solvents // XV Meeting of Young Chemical Engineers: Book of Abstracts / XV. susret mladih kemijskih inženjera: knjiga sažetaka / Ujević Andrijić, Željka; Vidak, Andrej (ur.). Zagreb: Hrvatsko društvo kemijskih inženjera i tehnologa (HDKI), 2024. str. 108-108 (poster presentation)

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17. Pavličević, Lana; **Jurčević Šangut, Iva**; Šamec, Dunja. The influence of freeze-drying, air-drying and oven-drying on the biflavones content of yellow ginkgo (*Ginkgo biloba* L.) leaves // Book of abstracts of 2nd international conference "Food and Climate Change" / Babić, Ivana; Šamec, Dunja; Sviličić Petrić, Ines (ur.). Koprivnica: Sveučilište Sjever, 2023. str. 88-88 (poster presentation)

18. **Jurčević, Iva**; Šarkanj, Bojan; Šamec, Dunja. The influence of 3',8" dimerization on the antioxidant and antifungal activity of flavonoids: the example of apigenin and amentoflavone // Book of abstracts; 10th International Congress of Food Technologists, Biotechnologists and Nutritionists. Zagreb: Hrvatsko društvo prehrambenih tehnologa, biotehnologa i nutricionista, 2022. str. 100-100 (poster presentation)

19. Sudarić Bogojević, Mirta; **Jurčević, Iva**; Cvitković, Ante; Valjetić, Marijana. Residential Backyards – Places for the Spread of Invasive Mosquito Species // Proceedings of the 10th International Conference on Urban Pests. Barcelona, 2022. str. 414-414 (poster presentation)

20. Sudarić Bogojević, Mirta; Cvitković, Ante; Valjetić, Marijana; **Jurčević, Iva.** A Five-Year Study on the Presence and Spread of Invasive Mosquito Species in Brod-Posavina County, Croatia // 4. hrvatski simpozij o invazivnim vrstama s međunarodnim sudjelovanjem. Zagreb: Hrvatsko ekološko društvo, 2021. str. 77-77 (poster presentation)

21. Sudarić Bogojević, Mirta; **Jurčević, Iva**; Cvitković, Ante; Valjetić, Marijana. Diversity and seasonal dynamics of invasive mosquito species in Brod-Posavina County, Croatia // Journal of the European Mosquito Control Association 39 Supplement 1. Beč: Wageningen Academic Publishers, 2021. str. 48-48 (poster presentation)

Projects:

1. Research assistant and on the Croatian Science Foundation project GinkoBiFlav (UIP-2019-04-1018) (29 June 2022 – 14 June 2026) (PI: Assist. Prof. Dunja Šamec, PhD)

2. Collaborator on the projects: Biotransformations and Biological Activity of Methylated Flavones and Their Dimers (UNIN-BIOTEH-23-1-1) (30 March 2023 – 31 March 2024, PI: Assist. Prof. Dunja Šamec, PhD), Biotransformations and Biological Activity of Methylated Flavones and Their Dimers – Second Research Phase (UNIN-BIOTEH-24-1-1) (25 March 2024 – 31 March 2025, PI: Assist. Prof. Dunja Šamec, PhD), GreenBioTECHNO: Green Biotechnology in Food Production and Processing (UNIN-BIOTEH-25-1-1) (1 April 2025 – 31 March 2026, PI: Assist. Prof. Dunja Šamec, PhD), Strategies for Mitigating Mycotoxin Contamination in Food and Feed (UNIN-BIOTEH-25-1-4) (18 April 2024 – 31 March 2026, PI: Marija Kovač Tomas, PhD), Novel Biotechnological Solutions in Climate Change Mitigation – BIOSHIELD (2024-1-EL01-KA220-HED-000251373) (1 October 2024 – 14 June 2026, Institutional PI: Assist. Prof. Dunja Šamec, PhD).

Workshops:

1. April 2024. DDD and ZUPP ’24 Community involvement - The importance of accurate identification of vectors and pests for timely and appropriate action in public health situations
2. April 2023. DDD and ZUPP ’23 Public health responsibility then and now - The public health responsibility of timely preparation for the invasion of the yellow fever mosquito*, Aedes aegypti*, into the territory of Croatia
3. November 2023. BIOCentar – Fundamentals of Liquid Chromatography (UHPLC)
4. December 2022. Ruđer Bošković Institute – Introduction to Molecular Modeling