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Use of natural extracts from *Lamium album* in the biological protection of cereals against *Fusarium* pathogens

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Poznań, 2024

Dedication

First and foremost, I would like to thank God for His protection and countless blessings.

Special gratitude to my beloved parents, Urayeneza Leocadie and Harindintwari Viateur, whose constant encouragement inspired me to confront each new day and challenge.

I am grateful to my husband, Nyandwi Xavier, who encouraged and supported me through all my difficulties.

Many thanks to my brother, Byamungu Desire, whose warm jokes, pizza invitations, and unwavering support gave me the energy I needed between my hard workdays.

I would like to thank my supervisor, Prof. dr hab. Agnieszka Waśkiewicz, for her consistent support, expertise, and passion for research that enabled me to follow my journey and become the person I am today.

Special thanks to Prof. dr hab. Jean Diatta for his moral support and guidance during my studies.

My thanks also go to Prof. dr hab. Lukasz Stepien and dr Monika Urbaniak for their support and assistance with the microbiological aspect of my research at the Polish Academy of Sciences' Institute of Plant Genetics. Your passion and strong research standards inspired me to embrace possibilities with innovative approaches.

My heartfelt thanks go to my friends and colleagues for the assistance they provided throughout my studies, especially my labmates from the Institute of Plant Genetics of the Polish Academy of Sciences, Plant-Pathogen Interaction Team, and the Institute of Agricultural and Food Biotechnology - State Research Institute, Department of Food Safety and Chemical Analysis for their generous support, patience, and dedication.

I would like to express my gratitude to my colleagues and co-authors for their valuable contributions, which helped me get my research works published in reputable journals.

Finally, I would also like to thank everyone who contributed morally or materially, directly or indirectly, to the successful completion of this work.

Acknowledgment

This research was funded by the NATIONAL SCIENCE CENTRE, POLAND, grant OPUS 16, number: UMO-2018/31/B/NZ9/03485.

List of publications included in the doctoral dissertation:

P1: **Uwineza, P.A.**; and Waśkiewicz, A. 2020. Recent Advances in Supercritical Fluid Extraction of Natural Bioactive Compounds from Natural Plant Materials. *Molecules* 25, 3847. https://doi.org/10.3390/molecules25173847. **IF=4.2, MEiN$_{2024}$=140**

P2: **Uwineza, P.A.**; Gramza-Michałowska, A.; Bryła, M.; and Waśkiewicz, A. 2021. Antioxidant Activity and Bioactive Compounds of *Lamium album* Flower Extracts Obtained by Supercritical Fluid Extraction. *Appl. Sci.* 11, 7419. https://doi.org/10.3390/app11167419. **IF= 2.5, MEiN$_{2024}$=100**

P3: **Uwineza, P. A.**; Urbaniak, M.; Stępień, Ł. ; Gramza-Michałowska, A. and Waśkiewicz, A. 2023. *Lamium album* Flower Extracts: A Novel Approach for Controlling *Fusarium* Growth and Mycotoxin Biosynthesis. *Toxins*, 15(11), 651. https://doi.org/10.3390/toxins15110651. **IF=3.9, MEiN$_{2024}$=100**

P4: **Uwineza, P. A**; Urbaniak, M.; Stępień, Ł.; Gramza-Michałowska, A. and Waśkiewicz, A. 2024. Efficacy of *Lamium Album* as a Natural Fungicide: Impact on Seed Germination, Ergosterol, and Mycotoxins in *Fusarium culmorum*-Infected Wheat Seedlings. *Front. Microbiol.* 5, 1363204. https://doi.org/10.3389/fmicb.2024.1363204. **IF=4.0, MEiN$_{2024}$=100**

P5: **Uwineza, P.A**.; Kwiatkowska, M.; Gwiazdowski, R.; Stępień, Ł.; Bryła, M.; Waśkiewicz, A. 2024. Field Assessment of *Lamium album* in Reducing Mycotoxin Biosynthesis in Winter Wheat Infected by *Fusarium culmorum*. *Agriculture,* 14, 647. https://doi.org/10.3390/agriculture14050647. **IF=3.3, MEiN$_{2024}$=100**

Total Impact Factor: **17.9**

Total points according to the list of MEiN journals for the publications: **540**

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The list of abbreviations

ABTS: 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid

ANOVA: analysis of variance

CTAB: hexadecyltrimethylammonium bromide

DNA: deoxyribonucleic acid

DON: deoxynivalenol

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EFSA: European Food Safety Authority

ERG: ergosterol

EC50: effective concentration 50%

FBs: fumonisins (B_1, B_2 and B_3)

FCR: Fusarium crown rot

FHB: Fusarium head blight

FUS-X: fusarenon X

FRAP: ferric reducing ability of plasm

FRR: Fusarium root rot

FSB: Fusarium seedling blight

GAE: gallic acid equivalents

GC-MS: gas chromatography-mass spectrometry

NIST: National Institute of Standards and Technology database

NIV: nivalenol

IPM: Integrated Pest Management

PCR: polymerase chain reaction

PDA: potato dextrose agar

SC-CO_2: supercritical carbon dioxide

SFE: supercritical fluid extraction

SLVI: seedling length vigor index

SWVI: seedling weight vigor index

TE: trolox equivalents

TPC: total phenolic content

UHPLC-HESI-MS/MS: ultra-high-performance liquid chromatography coupled with Q-exactive orbitrap mass spectrometry equipped with heated electrospray ionization source

UPLC-PDA-TQD: ultra-performance liquid chromatography coupled with photodiode array detector and triple quadrupole mass spectrometer

ZEN: zearalenone

ZEN-14S: zearalenone-14-sulfate

α-ZOL: alpha-zearalenol

β-ZOL: beta-zearalenol

3- and 15-AcDON: 3- and 15-acetyldeoxynivalenol

Summary

Fusarium pathogens are the primary cause of several diseases in cultivated plants, including wheat, and their secondary metabolites (mycotoxins) pose health hazards to humans and animals. In the age of sustainable agriculture, it is particularly important to search for environmentally friendly methods for preventing and combating plant diseases. Plant extracts as biocontrol agents have gained popularity as they are a rich source of bioactive compounds that can inhibit the growth of fungal pathogens. In the series of publications in this doctoral dissertation, an attempt was made to investigate the efficacy of natural plant extracts from native herbal plants in the biological protection of cereals against agriculturally important *Fusarium* pathogens - *Fusarium culmorum* and *Fusarium proliferatum*. For this purpose, multidirectional studies were carried out, starting from the development of extraction conditions using a modern technique based on supercritical carbon dioxide (SC-CO_2) and selecting one potential plant from four families (*Lamiaceae, Rosaceae, Ranunculaceae, and Elagnaceae*) - based on the obtained results (the effect of extraction parameters on the extracts composition and their antioxidant activity, extraction efficiency, composition and concentration of bioactive compounds) - with promising antifungal potential: *Lamium album*. Following this, the antifungal properties of *L. album* flower extracts against *Fusarium* pathogens were evaluated both *in vitro* (PDA medium) and *in vivo* (wheat cultivation in pots and the field) experiments. These investigations provided insights into the effectiveness of the extracts in preventing fungal growth, lowering the mycotoxins biosynthesis, and assessing any potential harmful impacts of the extract on wheat germination and seedling growth. The results demonstrated the potential of *L. album* flower extract obtained by SC-CO_2 as a natural source of bioactive compounds with different biological activities, including antifungal activity. Its potential efficacy in suppressing *Fusarium* growth from *in vitro* to *in vivo* was observed and confirmed with the reduction in ERG and mycotoxin biosynthesis. These findings – described for the first time in the literature – contribute to the development of research into alternative, biological solutions to synthetic fungicides and emphasize the importance of conducting comprehensive studies before practical application in the agricultural environment.

Keywords: *Lamium album*; supercritical fluid extraction; plant extracts; *Fusarium* pathogens; mycotoxins, ergosterol; antifungal activities; sustainable agriculture

Streszczenie

Grzyby rodzaju *Fusarium* są przyczyną wielu chorób roślin uprawnych, w tym pszenicy, a ich produkty metabolizmu wtórnego (mykotoksyny) stanowią zagrożenie zdrowotne dla ludzi i zwierząt. W dobie zrównoważonego rolnictwa szczególnie istotne jest poszukiwanie przyjaznych dla środowiska metod zapobiegania i zwalczania chorób roślin. Wykorzystanie ekstraktów roślinnych w biologicznej kontroli zyskuje rosnącą popularność z racji bogactwa substancji bioaktywnych mogących hamować rozwój patogenów grzybowych. W cyklu publikacji wchodzących w skład rozprawy doktorskiej podjęto próbę zbadania skuteczności naturalnych ekstraktów roślinnych z pochodzących rodzimych roślin zielnych w biologicznej ochronie zbóż przed ważnymi rolniczo patogenami *Fusarium* - *Fusarium culmorum* i *Fusarium proliferatum*. W tym celu przeprowadzono wielokierunkowe badania, począwszy od opracowania warunków ekstrakcji przy użyciu nowoczesnej techniki bazującej na wykorzystaniu dwutlenku węgla w stanie nadkrytycznym (SC-CO_2) i wytypowaniu na podstawie uzyskanych wyników (wpływu parametrów ekstrakcji na skład ekstraktów i ich aktywność antyoksydacyjną, wydajność ekstrakcji, kompozycja i steżenie związków bioaktywnych) spośród 4 rodzin (*Lamiaceae*, *Rosaceae*, *Ranunculaceae* i *Elagnaceae*) jednej rośliny o obiecującym potencjale przeciwgrzybiczym - *Lamium album*. Następnie oceniono właściwości przeciwgrzybicze ekstraktów z kwiatów *L. album* względem *Fusarium* zarówno w doświadczeniach *in vitro* (pożywka PDA), jak i *in vivo* (uprawa pszenicy w donicach i na poletkach). Badania te dostarczyły wglądu w skuteczność ekstraktów w zapobieganiu wzrostowi grzybów *Fusarium*, obniżaniu biosyntezy mykotoksyn i ocenie wszelkich potencjalnie szkodliwych skutków wpływu ekstraktu na kiełkowanie pszenicy. Wyniki wykazały potencjał ekstraktów z kwiatów *L. album* pozyskanych metodą SC-CO_2 jako naturalnego źródła związków bioaktywnych o różnych aktywnościach biologicznych, w tym aktywności przeciwgrzybiczej. Jego potencjalna skuteczność w hamowaniu wzrostu grzybów *Fusarium* w doświadczeniach *in vitro* i *in vivo* została zaobserwowana i potwierdzona redukcją biosyntezy ERG i mykotoksyn. Odkrycia te – po raz pierwszy opisane w literaturze - przyczyniają się do rozwoju badań nad alternatywnymi, biologicznymi rozwiązaniami względem syntetycznych fungicydów i podkreślają ważność przeprowadzenia kompleksowych badań przed praktycznym zastosowaniem w środowisku rolniczym.

Słowa kluczowe: *Lamium album*; ekstrakcja płynem nadkrytycznym; ekstrakty roślinne; patogeny *Fusarium*; mykotoksyny; ergosterol; aktywność przeciwgrzybicza; zrównoważone rolnictwo

1. Introduction

In recent years, the quest for sustainable agricultural practices has led to a growing interest in utilizing natural plant extracts to safeguard cereals against *Fusarium* pathogens. This trend arises from emerging concerns over the adverse effects of synthetic fungicides on both human health and the environment. While the Green Revolution brought about remarkable advancements in crop cultivation through the widespread adoption of agrochemicals, it also brought to light the detrimental repercussions of pesticide usage. Extensive documentation since the 1963s underscores the detrimental impacts of pesticides on human health, wildlife, and ecosystems (Besset-Manzoni et al., 2019). Furthermore, studies have shown that pesticides, including synthetic fungicides, exhibit limited efficacy, with only a fraction of 0.1 to 0.3% of applied chemicals reaching their intended targets, leaving the majority to disperse into the environment (Banaszkiewicz, 2010; Pimentel, 1995). Consequently, this indiscriminate dispersion contributes to the development of resistance among phytopathogens, further exacerbating the challenge of pest control. In response, integrated pest management (IPM) strategies have emerged, emphasizing the cultivation of healthy crops while minimizing disruption to agroecosystems and promoting natural pest control mechanisms in agriculture (Barzman et al., 2015).

As the primary source of essential food resources, agriculture plays a pivotal role in sustaining and nourishing societies while contributing significantly to economic growth and overall development. With a global population approaching nearly 10 billion by 2050, the need to enhance agricultural productivity becomes increasingly urgent, especially considering the limited availability of arable land (Ortiz et al.,2021). Cereals, being fundamental staples, play a pivotal role in addressing these challenges of meeting the growing demand for food. However, these crucial crops face substantial threats from phytopathogenic fungi, contributing significantly to global food losses and compounded by climate change and environmental issues (Singh et al., 2023). The fungal infections contribute to substantial losses, accounting for approximately 70–80% of agricultural production losses (Peng et al., 2021).

Phytopathogenic fungi, particularly those of the *Fusarium* genus, are a group of filamentous ascomycete fungi that pose substantial diseases to cereals throughout various growth stages, from the field to post-harvest and even during storage (Deresa & Diriba, 2023). *Fusarium* infection may start either in the roots due to inoculum or the soil-borne

spores or on the above-ground parts of the crop, introduced through various ways such as water, air, or agricultural equipment (Alisaac et al., 2023; Karlsson et al., 2021; Seepe et al., 2021). Pathogens can also infiltrate crops through injuries caused by insects and/or nematodes, however, various factors, including geographical location, genetic characteristics, and environmental conditions, influence the spread and development of *Fusarium* diseases (Popovski & Celar, 2013; Mielniczuk & Skwaryło-Bednarz, 2020), with temperature and water activity being the most significant conditions for *Fusarium* growth (Da et al., 2013; Popovski & Celar, 2013).

Fusarium fungal pathogens such as *Fusarium culmorum* and *Fusarium proliferatum* are prominent threats, profoundly impacting yield and quality. In cereals, *Fusarium* infections result in *Fusarium*-damaged kernels, spikelet sterility, reduced nutritional content, and the biosynthesis of toxic secondary metabolites - mycotoxins, posing detrimental effects on both human and animal health (Bota et al., 2021). Mycotoxins are synthesized both before and following harvest, with concentrations possibly increasing during post-harvest handling and storage (Da et al., 2013) due to various factors, including inadequate moisture and temperature control, prolonged storage durations, insect and rodent infestations or lack of proper aeration.

F. culmorum is a soil, air, and seed-borne fungus with a wide distribution and robust saprophytic capabilities (Antalová et al., 2020). It is a primary pathogen in wheat and is responsible for causing Fusarium crown rot (FCR), Fusarium seedling blight (FSB), and Fusarium root rot (FRR). However, Fusarium head blight (FHB) represents a far more significant threat (Antalová et al., 2020; Bottalico & Perrone, 2002; Scherm et al., 2012). Moreover, *F. culmorum* is recognized for its ability to synthesize a wide range of mycotoxins, including type B trichothecenes (mainly deoxynivalenol–DON with derivatives, 3- and 15-acetyldeoxynivalenol - 3- and 15-AcDON or nivalenol–NIV), zearalenone (ZEN) and its derivatives including beta-zearalenol (β-ZOL), alpha-zearalenol (α-ZOL) and zearalenone-14-sulfate (ZEN-14S) (Alisaac et al., 2023; Scherm et al., 2012). Additionally, ZEN and DON are the most prevalent mycotoxins found in commodities and grains contaminated by *F. culmorum*. ZEN, categorized as a phenolic resorcylic acid lactone mycotoxin, exerts strong estrogenic effects by binding to estrogen receptors, thereby impacting the reproductive functions of animals (Bertero et al., 2018). In contrast, DON disrupts cellular functions by binding to the ribosome, inhibiting protein synthesis (Scherm et al., 2012). It has been

implicated in inducing apoptosis (Desmond et al., 2008) and plays a significant role as the virulence factor (Bai et al., 2002).

On the other hand, *F. proliferatum* is primarily linked to maize contamination as a pathogenic fungus. Although commonly acknowledged as a field pathogen, it can also grow in storage environments characterized by low temperatures and high moisture content (Gálvez et al., 2020; Mondani et al., 2021; Velluti et al., 2003). The primary modes of dissemination and propagation for *F. proliferatum* involve seeds and crop residues. Initial infections during the seedling stage can lead to plant mortality, while later-stage infections often result in diminished yields. *F. proliferatum* is notably proficient in producing various mycotoxins, with fumonisin B1 being a significant example (Guo et al., 2018). This mycotoxin is categorized as a group B carcinogen in humans by International Agency for Research on Cancer (IARC, 2002) and has been associated with conditions such as oesophageal and liver cancers (Domijan, 2012; Seo & Yu, 2004; Sydenham et al., 1990; Wild & Gong, 2010), as well as neural tube defects (Domijan, 2012).

Given these challenges, protecting cereals, particularly wheat, against *Fusarium* spp. and their toxic secondary metabolites becomes paramount for global food security. This is because wheat is widely recognized as one of the essential staple foods globally for various reasons (Reynolds & Braun, 2022), such as its versatility in culinary applications, nutritional richness, and global dietary significance that make it a fundamental component of diverse diets (Iqbal et al., 2022; Reynolds & Braun, 2022). Furthermore, with its adaptability to produce a range of food products, including bread, pasta, cereals, and beer, wheat has become a dietary cornerstone for numerous cultures worldwide (Shewry & Hey, 2015). Its economic importance as a cash crop further solidifies its role in sustaining livelihoods and contributing to economies. Therefore, its protection is imperative for preserving its role as a staple food, maintaining its nutritional value, economic viability, and contribution to global food security.

Conventionally, several management strategies, including crop rotation, the use of genetically resistant seeds and synthetic fungicides, have been employed with varying success (Alisaac et al., 2023; Cunfer, 1994; Özdemir, 2022; Wegulo et al., 2015). Briefly, synthetic fungicides have been one of the primary methods for protecting wheat against phytopathogens due to their effectiveness in eliminating pathogens, reducing their spread, and inhibiting disease progression (Zubrod et al., 2019). However, their excessive and prolonged use poses risks to human health and the ecosystem (Da et al., 2013). For instance,

some fungicides, like methyl bromide and carbendazim, have been reported to be toxic, have limited biodegradability, and affect non-targeted organisms (Seepe et al., 2021). Additionally, certain fungi have developed resistance to specific fungicides, reducing their effectiveness due to repeated use and increased doses (Castro et al., 2020; Seepe et al., 2021). Therefore, it is crucial to investigate and develop new methods or alternatives to fight against *Fusarium* pathogens on cereals to enhance understanding of integrated management systems and implement knowledge-based plant protection measures. This is essential for reducing reliance on synthetic fungicides through efficient natural biological methods as part of the transition toward sustainable crop cultivation.

One promising approach is using plant extracts, which have evolved as potential alternatives to synthetic fungicides (Abdallah et al., 2018). This shift is driven not only by the pursuit of sustainable development and the ecological safeguarding of nature, wildlife, and agricultural commodities (Da et al., 2013) but also by the recognition of this innovative approach as effective, environmentally friendly, biodegradable and economically practical (Mahlo et al., 2016; Suteu et al., 2020; Velluti et al., 2003). Plant extracts encompass substances derived from various parts of plants, including roots, barks, seeds, shoots, leaves, fruits, flowers, cloves, rhizomes, or stems that offer the advantage of containing a blend of various compounds, including phenolic acids, flavonoids, tannins, terpenes, and alkaloids (Acheuk et al., 2022), that can synergistically collaborate or individually provide several biological activities including the inhibition of phytopathogenic fungi growth in the field and post-harvest (Deresa & Diriba, 2023). In addition, various mechanisms of action of the plant extracts against phytopathogenic fungi have been reported, including interference with oxidative balance, disruption of the mitochondrial structure, increased cell membrane permeability, reduction of ergosterol content, and inhibition and degradation of mycotoxin biosynthesis (Acheuk et al., 2022; Jafarzadeh et al., 2022).

Currently, numerous studies have highlighted the antifungal effects of various plant extracts, including *Melissa officinalis* (Uwineza et al., 2022), *Cymbopogon citratus*, *Lantana camara*, *Ocimum basilicum*, and *Zingiber officinales* (Ahmed et al., 2023), *Ambrosia confertiflora, Azadirachta indica, Baccharis glutinosa, and Larrea tridentata, Urtica dioica* (Hadizadeh et al., 2009), *Glechoma hederacea var. longituba* (Gwiazdowska et al., 2022) against different *Fusarium* species, including *Fusarium oxysporum, F. solani, F. culmorum, F. proliferatum, F. verticillioides, F. avenaceum, F. graminearum, and F. sporotrichioides*

(Ahmed et al., 2023; Aziz & Al-Askar, 2012; Kursa et al., 2022; Suárez-Jiménez et al., 2007; Uwineza et al., 2022). However, most of these studies were conducted *in vitro* and often neglect the impact of plant extracts on mycotoxin biosynthesis - a crucial outcome of fungal infection that affects both the quality of the produce and the health of consumers. Moreover, the literature showed that the number of biopesticides based on plant extracts that have undergone testing remains limited, and there is an increasing demand for organic products.

One of the plants - with unexplored antimicrobial potential - and having several health properties is *Lamium album*, commonly known as white dead nettle or non-stinging nettle. It is a medicinal herbaceous plant from the *Lamiaceae* family indigenous to Europe, Western Asia, and North Africa (Bubueanu et al., 2013; Yordanova et al., 2014). Its young shoots, leaves, and flowers are edible, whether consumed fresh or cooked (Pereira et al., 2012). In contemporary usage, *L. album* is employed in producing beverages like tea and dietary supplements, with claims of detoxifying the organism and preventing conditions such as menstrual disorders, abdominal inflammation, and musculoskeletal diseases (Pereira et al., 2012; Yalçin, 2006). *L. album* has been studied through various *in vitro* and *in vivo* model systems, revealing its pharmacological and biological effects such as antiviral, antibacterial, antioxidant, anticancer, cytoprotective, and wound-healing properties (Chipeva et al., 2013; Czerwińska et al., 2020; Moskova-Doumanova et al., 2012; Paduch et al., 2007; Shah et al., 2019; Todorov et al., 2013). The diverse biological activities of *L. album* are attributed to its various biologically active compounds, such as iridoids (lamalbid), phenolic acids (chlorogenic acid, gallic acid, caffeic acid, and syringic acid), phenylpropanoids (verbascoside), and flavonoids (rutin, quercetin malonylhexoside, tiliroside). These compounds have been identified in different extracts of *L. album*, including ethanolic-aqueous and methanol extracts (Czerwińska et al., 2020; Paduch et al., 2007; Sulborska et al., 2020). Although there have been different studies on the pharmacological properties of *L. album*, there has been little focus on studying *L. album* flower extracts as potential natural antifungal agents in agriculture. Moreover, there is a significant lack of research investigating the effectiveness of *L. album* flower extracts against *Fusarium* pathogens *in vivo*.

Typically, extracts are obtained through conventional extraction techniques such as steam distillation, hydro-distillation, and solvent extraction. It is important to note that, despite the absence of a universally acknowledged standard method for plant-based extracts (Azmir et al., 2013), the choice of extraction method holds significant importance. The

appropriate selection of an extraction technique can result in a high content of biologically active compounds within the extract, promoting its biological activities. Nevertheless, conventional techniques present several drawbacks, including extended extraction times, the need for costly and highly pure solvents, loss of volatile compounds, low yield, poor extraction selectivity, and the degradation of thermally labile compounds (Azmir et al., 2013; Ghasemi et al., 2011). Therefore, innovative and promising extraction techniques have been introduced to address these limitations associated with conventional extraction methods. One such method is supercritical fluid extraction (SFE), which has emerged as a non-conventional approach for obtaining plant extracts rich in various bioactive compounds due to their unique properties (Bimakr et al., 2011).

Supercritical fluid extraction with CO_2 (SC-CO_2) is an efficient and environmentally friendly method for isolating secondary metabolites from plants by leveraging the unique characteristics of fluids at their critical points. In the supercritical state, solvents exhibit properties of both liquids and gases, such as low viscosity, high diffusivity, and density, which enhance their effectiveness in extraction processes (Raventós et al., 2002). Additionally, CO_2 is the preferred solvent in SFE due to its relatively low critical pressure (74 bar) and temperature (32°C). This choice is justified by its cost-effectiveness, wide availability, non-toxicity, non-flammability, and ease of removal from the final extract while preserving the biological properties of the extracted compounds (Pourmortazavi & Hajimirsadeghi, 2007). By adequately controlling SC-CO_2 parameters, especially pressure and /or temperature, the extractability property can also be modified, enabling this process to find its field in the extraction of natural plant compounds.

Therefore, the primary goal of this study was to assess the effectiveness of natural plant extracts from native herbal plants, particularly *L. album*, in protecting cereals against *Fusarium* pathogens. The study integrated various experimental approaches (as presented in Figure 1), including screening various plants using SC-CO_2 extraction, conducting *in vitro* and *in vivo* trials to evaluate the chemical composition of *L. album* flower extracts, efficacy, and practical applicability of *L. album* flower extracts to fight against *Fusarium culmorum* and *F. proliferatum*. The results demonstrated the promising antifungal activity of *L. album* flower extract against the studied *Fusarium* pathogens. These findings are critical for advancing scientific understanding of the potential of plant extracts in combating plant pathogens, as well as the development of future commercial products and environmentally

friendly farming strategies. The study contributes to ongoing research on exploring potential plants with fungicidal properties, developing effective extraction methods, and effective strategies for protecting cereals against *Fusarium* infections.

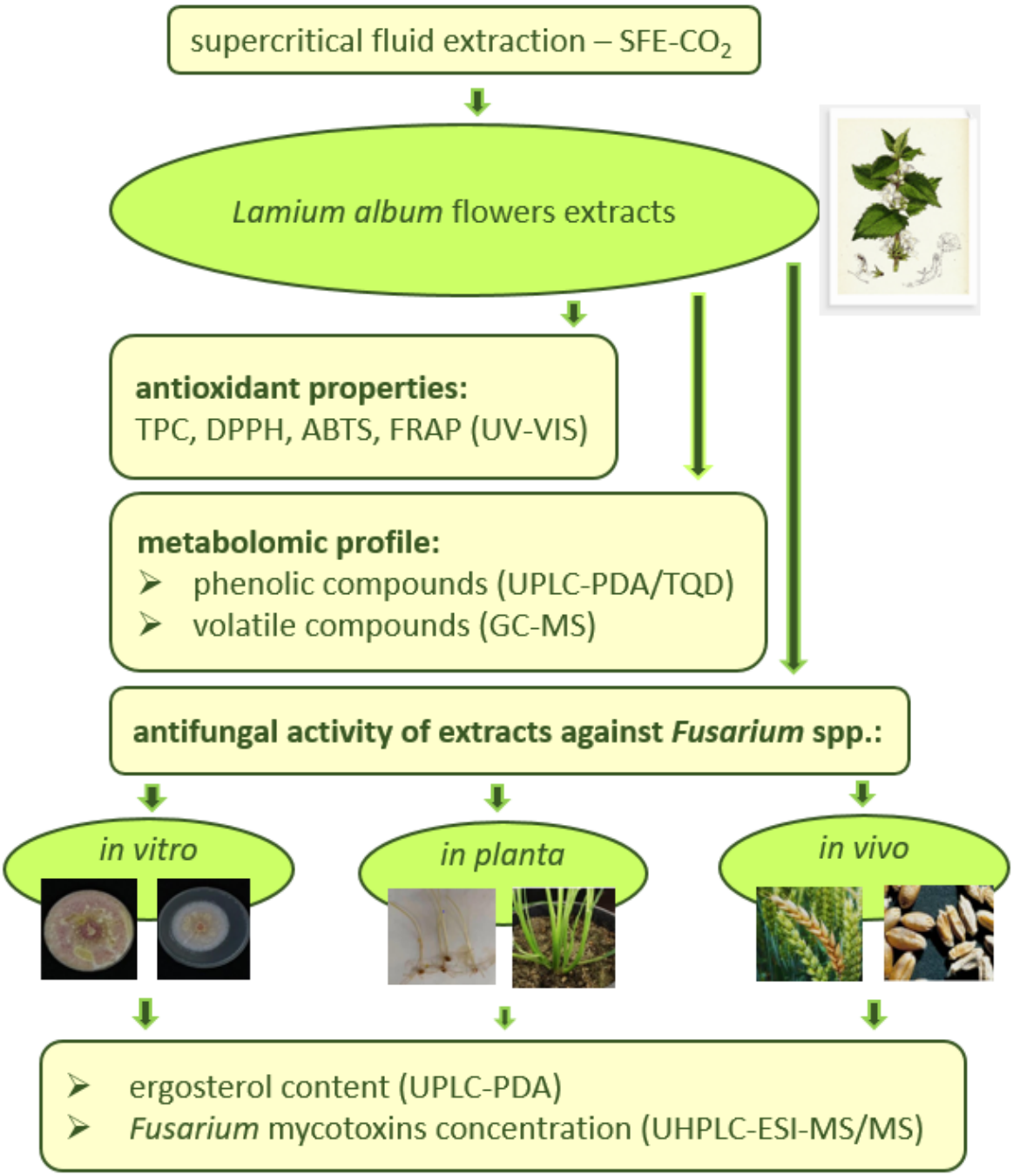


Figure 1. Diagram of the research model with methodology

2. Research goals and hypothesis

2.1. Research goals

This research aimed to assess the efficacy of natural extracts from native herbal plants, specifically *Lamium album* flowers, in the biological protection of cereals against *Fusarium* pathogens.

The main goal was achieved by defining and realizing the specific objectives:

- To develop and optimize the extraction conditions for *L. album* flowers using supercritical carbon dioxide (SC-CO_2) with methanol as a co-solvent, with the evaluation of the effects of temperature, pressure, CO_2 flow rate, and extraction time on yield and extract composition (P1 and P2)
- To characterize the bioactive compounds in *L. album* flower extracts by analyzing their volatile and phenolic compounds, as well as assessing their antioxidant activity, to comprehensively establish a detailed metabolomic profile of the extracts and evaluate their antioxidant capabilities (P2)
- To assess the *in vitro* antifungal activity of *L. album* flower extracts at different concentrations in inhibiting the mycelia growth of selected *Fusarium* species *(F. culmorum* and *F. proliferatum)* and their impact on mycotoxin biosynthesis (P3)
- To investigate the phytotoxic impact of *L. album* flower extracts at various concentrations on wheat seed germination and seedling growth to gain an insight into the potential adverse effects of the extracts on wheat intended to protect (P4)
- To evaluate the *in vivo* antifungal potential of *L. album* flower extracts against *F. culmorum* infections in wheat under controlled growth conditions and field experiments on selected winter wheat cultivars (Julius and Arkadia) (P4 and P5).

2.2. Research hypothesis

In this research, the following research hypotheses were verified:

H1: The volatile and phenolic compounds and antioxidant activity of *L. album* flower extracts are significantly influenced by the SC-CO_2 extraction conditions, including temperature, pressure, CO_2 and co-solvent flow rates, and extraction time. Optimizing these parameters enhances the quality of the extracts.

H2: The *in vitro* antifungal activity of *L. album* flower extracts against *Fusarium culmorum* and *Fusarium proliferatum* depends on the extract concentration and *Fusarium* species.

H3: *L. album* flower extracts may exhibit phytotoxic effects on wheat seed germination and seedling growth, with different levels of impact depending on the extract concentrations, thus providing insights into the safe application levels for biological control in agricultural settings.

H4: The application of *L. album* flower extracts may demonstrate *in vivo* antifungal potential against *F. culmorum* infections in wheat seedlings under controlled growth conditions and field trials with winter wheat cultivars by potentially inhibiting fungal infections and mycotoxin biosynthesis.

3. Methods

The study examined:

- Plant material characteristics: dried *Lamium album* flowers from the *Lamiaceae* family were purchased from a certified Polish company called Dary Natury located in Podlaskie Voivodeship of Poland (53°4’10.98 latitude and 22°58’2.87 longitude). The selection of this plant was guided by trial screenings for antioxidant and antifungal properties. Before the extraction, the dried *L. album* flowers were finely ground to achieve a uniform particle size.

- Plant extraction using SC-CO_2 extraction: the extraction process was carried out using a supercritical fluid extractor (MV-10ASFE system, Waters, Manchester, MA, USA). The parameters tested included: temperature (40-60°C) and pressure (250-300 bars), CO_2 flow rate, co-solvent, and extraction time.

- Antioxidant activity was assessed using the DPPH assay (Moradi et al., 2016), ABTS assay (Re et al., 1999), and FRAP assay (Benzie & Devaki, 2017). TPC was determined using Folin–Ciocalteu assay (Arabshahi-Delouee & Urooj, 2007; Li et al., 2007; Mabrouki et al., 2018). All assays were conducted using a spectrophotometric method with a Varian Cary 300bio UV-VIS spectrophotometer, measuring absorbance at 517, 734, 593, and 760 nm, respectively.

- Metabolomic profiling of the extract: phenolic compounds were analyzed using UPLC/PDA-TQD and volatile compounds were evaluated using GC-MS.

- The *in vitro* antifungal activity of *L. album* flower extracts was assessed on a PDA medium by the poisoned food method (Abhishek et al., 2015; Gakuubi et al., 2017).

- DNA extraction and identification were performed using the PCR method (Stepień & Waśkiewicz, 2013; Urbaniak et al., 2020).

- Ergosterol analysis was conducted using the chromatographic method - UPLC-PDA-TQD (Waśkiewicz et al., 2014).

- Mycotoxins analysis was performed using the chromatographic method - UHPLC-HESI-MS/MS.

- Statistical analysis was conducted using Statgraphics 4.1 software package (Graphics Software System, STCC, Inc., Rockville, MD, USA), Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA), and GraphPad Prism 9 software.

4. The most important results with the discussions

In the first stage of this research, an extensive evaluation in terms of extraction efficiency, antioxidant properties, and antifungal activity was conducted on selected plants from 4 families - *Lamiaceae, Rosaceae, Ranunculaceae,* and *Elaeagnaceae* to determine the main plant for the research. The plant species assessed included *Melissa officinalis* (herbs, *Lamiaceae*), *Potentilla erecta* (rhizome, *Rosaceae*), *Lamium album* (flowers, *Lamiaceae*), *Hippophae rhamnoides* (leaves and fruits, *Elaeagnaceae*), *Crataegus monogyna* (leaves and fruits, *Rosaceae*), *Nigella sativa* (seeds, *Ranunculaceae*), *Sanguisorba officinalis* (herbs, *Rosaceae*), *Cimicifuga racemosa* (leaves, *Ranunculaceae*), *Stachys sylvatica* (herbs, *Lamiaceae*), *Glechoma hederacea* (leaves, *Lamiaceae*), and *Hyssopus officinalis* (herbs, *Lamiaceae*) that were selected due to their well-known medicinal properties and their potential as a source of bioactive compounds, which are essential for antioxidant and antifungal applications. The SC-CO_2 extraction method was used to extract these plants, in which various variable parameters were tested such as temperature, pressure, CO_2 flow rate, extraction time, and methanol flow rate as a co-solvent to assess their impact on extract composition, as well as to analyze the *in vitro* antioxidant and antifungal properties of each plant as part of the screening process.

Among the plants tested, *Lamium album* proved to be the most promising, with the highest antioxidant and antifungal properties, which led to its selection as the primary plant for the research. This choice was further supported by its broad availability and affordability in Poland (Jędras & Maciejewska-Rutkowska, 2007; Sulborska et al., 2014). Additionally, the limited research on *L. album* flowers, particularly regarding their antifungal properties, highlighted the importance of this research and its potential to increase the valorization of locally available plant resources in Poland. Therefore, the most significant findings of this research, selected from the four published papers, are presented and discussed in the following sections to provide an overview of the key results and their implications.

4.1. Extracts analysis (P2)

4.1.1. Impact of extraction conditions on antioxidant activity and chemical composition

Dried *L. album* flowers were extracted using the SFE with various parameters, including three different temperatures (40, 50, and 60°C), pressure (250 and 300 bars), SC-CO_2 as the solvent, and methanol as a co-solvent with dynamic and static modes. This

technique is well-known for its environmentally friendly nature and high efficiency in isolating bioactive compounds, making it an excellent alternative to existing extraction methods (Ahmad et al., 2019; Raventós et al., 2002). Compared to conventional methods like Soxhlet extraction, steam distillation, and organic solvent extraction, SC-CO_2 produces concentrated extracts that are often richer in antioxidant and antifungal compounds (Bai et al., 2020; Bimakr et al., 2011; Suetsugu et al., 2013). The selection of SC-CO_2 was based on its non-toxic and non-explosive characteristics (Benito-Román et al., 2018) and its selectivity as it can be easily changed by changing the pressure and temperature, which makes it perfect for extracting a wide range of chemicals.

However, due to its non-polar nature, SC-CO_2 alone did not effectively extract polar compounds like phenolics then methanol was used as a co-solvent to enhance the solvation power of SC-CO_2, improve its affinity for poorly soluble solutes, increase solubility, and improve extraction efficiency. Unlike water or ethanol, which can be used as co-solvents, methanol can easily penetrate plant matrices and dissolve a broader range of bioactive compounds, often challenging to extract with SC-CO_2.

To comprehensively assess the effect of SC-CO_2 parameters, particularly temperatures (40, 50, and 60°C) on antioxidant activity, and phenolic profile of *L. album* flower extracts, the results presented in Table 1 were evaluated.

Table 1. Antioxidant activities of *L. album* flower extracts extracted using SC-CO_2 with methanol at different temperatures

variants	TPC (mg GAE/g)	DPPH EC_{50} (mg/ml)	ABTS (µg TE/g)	FRAP (µmol TE/g)
40°C/250 bar	$234.17^{c} \pm 2.48$	$0.37^{a} \pm 0.04$	$43.20^{a} \pm 0.20$	$19.48^{c} \pm 0.05$
50°C/250 bar	$650.17^{a} \pm 3.86$	$0.12^{b} \pm 0.00$	$44.53^{a} \pm 0.02$	$44.74^{a} \pm 0.07$
60°C/250 bar	$418.50^{b} \pm 2.92$	$0.16^{b} \pm 0.00$	$44.52^{a} \pm 0.02$	$28.32^{b} \pm 0.12$

Notes: Each data point represents the mean ± SD of three independent replicates. The superscripts of different letters in rows are significantly different at $p < 0.05$ based on the Post hoc Tukey test.

These results were compared to understand how temperature influences the efficiency and effectiveness of the SC-CO_2 extraction process and by analyzing the obtained extracts at these different temperatures, the optimal conditions that maximized the extraction of bioactive compounds while minimizing degradation were identified. This comparison also

allowed us to observe how temperature variations affect the antioxidant activity of the extracts, ultimately guiding us to determine the most potent and beneficial conditions.

The TPCs in the obtained *L. album* flower extracts were determined quantitatively using a spectrophotometric method with Folin–Ciocalteu reagent (Arabshahi-Delouee & Urooj, 2007; Li et al., 2007; Mabrouki et al., 2018) as the most commonly used method for total phenols estimation; in addition, TPCs were evaluated to gain a more comprehensive understanding of the composition and antioxidant potential of *L. album* flower extracts as phenolic compounds are recognized to contribute significantly to the antioxidant capabilities of many plants. Further, the antioxidant activity was tested spectrophotometrically using three different assays-DPPH assay (Moradi et al., 2016), ABTS assay (Re et al., 1999), and FRAP assay (Benzie & Devaki, 2017) - to have a better estimation of the antioxidant capacity of the *L. album* flower extracts as a single method may not fully capture the diverse mechanisms of antioxidant characteristics.

The results in Table 1 show that the extracts obtained at a temperature of 50°C had the highest TPC with a value of 650.17 mg GAE/g. These extracts also exhibited the highest level of antioxidant activity in all tested assays. More precisely, the extracts showed the strongest DPPH radical scavenging activity, with an EC_{50} value of 0.12 mg/ml, indicating that a lower concentration of the extract was needed to achieve a 50% reduction in DPPH radicals, signifying a higher level of antioxidant potential. Additionally, the same extract demonstrated the highest ABTS activity, measuring 44.53 µg TE/g, and the strongest ferric-reducing antioxidant potency, with a FRAP value of 44.74 µmol TE/g. In contrast, the extracts obtained at a temperature of 40°C had the lowest TPC and antioxidant activity, whereas those obtained at 60°C exhibited intermediate values. Based on these findings, it is clear that the extraction temperature significantly influenced both the total phenolic content and antioxidant activities of *L. album* flower extracts. At the constant pressure (250 bars) used, the dual effect of temperature toward the extraction process was observed, which could be due to the interaction between the vapor pressure of the solute and the density of the solvent (Ahmad et al., 2019). From 40 to 50°C, the TPC and antioxidant activity increased significantly due to increased solute volatility, better diffusivity, and solvent density, all contributing to efficient extraction. However, from 50 to 60°C, a decrease in TPC and antioxidant activity was observed, but the values remained higher than at 40°C. This decline can be attributed to a substantial reduction in solvent density, which reduced the solvating

power of SC-CO_2 and the potential thermal degradation of phenolic compounds and antioxidants. As a result, the optimal extraction temperature for *L. album* flowers in this study was 50°C, as it exhibited the highest capacity for scavenging free radicals, the strongest reducing capability, and the total phenols, confirming the hypothesis that antioxidant activity and phenolic content of the plant extract are strictly dependent on the extraction condition used.

The correlations between the TPC and the antioxidant assays, including DPPH, ABTS, and FRAP, were analyzed to understand the relationship between phenolic compounds and antioxidant activity in *L. album* flower extracts. Significant positive correlations between TPC and each of the antioxidant assays were found, with the strongest correlation observed between TPC and FRAP (r=0.9941), followed by DPPH (r=0.7974) and ABTS (r=0.6271). These findings proved the essential effects of phenolic compounds in the antioxidant capacity of *L. album* extracts, with a powerful influence on reducing properties, as indicated by the highest correlation with FRAP. Moreover, the moderate to strong correlations between TPC, DPPH, and ABTS assays further support the notion that phenolic compounds contribute significantly to the scavenging of free radicals in these extracts.

4.1.2. Metabolomic profiling of L. album extracts using chromatographic techniques

In addition to spectrophotometric analyses, metabolomic profiling of *L. album* flower extracts was conducted to identify and quantify bioactive compounds using advanced chromatographic techniques, including UPLC-PDA-TQD for phenolic compounds and GC-MS for volatile compounds. These approaches, which are cost-effective, reproducible, accurate, and resilient, were used to characterize the bioactive compounds in these extracts obtained by SC-CO_2 extraction.

Using UPLC-PDA-TQD, an advanced analytical approach for phenolic compound identification and quantification, 13 phenolic compounds were successfully identified and quantified in *L. album* flower extracts out of 22 compounds tested, including— myricetin, quercetin, rutin, caffeic acid phenethyl ester, apigenin, pinostrobin, galangin, chrysin, vanillic acid, syringic acid, trans-3-hydroxycinnamic acid, trans-cinnamic acid. Three main phenolic groups were categorized based on their structure such as flavonoids, phenolic acids, and phenolic ester which highlights the diversity of bioactive compounds in *L. album* flower extracts and their potential roles in antioxidant and antifungal activities. The concentrations

of these compounds varied depending on the extraction conditions, with trans-cinnamic acid ranging from 0.09 ng/μL at 40°C to chrysin reaching 22.68 ng/μL at 50°C. Notably, the extracts obtained at 50°C indicated the highest level of chrysin, myricetin, pinostrobin, and *trans*-3-hydroxycinnamic acid. Significant differences ($p < 0.05$) were observed between the extraction conditions, with the extracts obtained at 50°C showing higher levels of most bioactive compounds than the extracts at 40°C. However, at the highest extraction temperature of 60°C, the concentration of most bioactive compounds decreased significantly compared to the 50°C extraction, with the exceptions of rutin, quercetin, and *trans*-cinnamic acid. These differences can be related to the influence of temperature on the solubility of phenolic compounds and the stability of bioactive compounds during the SC-CO_2 extraction process. While 50°C was considered the optimal condition in balancing solubility and preserving compound integrity, lower temperatures (40°C) resulted in reduced extraction efficiency, and higher temperatures (60°C) led to thermal degradation or structural alteration of some phenolics, thereby reducing their concentrations in the extracts.

Furthermore, the GC-MS system was used to analyze the volatile compounds in *L. album* flower extracts obtained under varying temperatures and pressures during SC-CO_2 extraction. GC-MS parameters were set to ensure comprehensive profiling. The mass spectrometer was operated in full scan mode with a scan range of m/z 30 to 450, enabling the detection of a wide array of compounds. The results revealed at least a total of seventy-two phytochemical compounds in the *L. album* flower extracts that likely contributed to the observed biological activities. The identified compounds were confirmed based on the peak area, retention time, and molecular formula, with compound prediction facilitated by the NIST Database.

These compounds were categorized into various functional groups with different peak areas including sesquiterpenes ranging from 7.54 to 9.90%, monoterpenes (0.75-3.07%), alcohols (53.37-54.22%), ketones (27.92-30.71%), phenols (0.31-0.62%), phenylpropanoids (4.46-5.08%), aldehydes (0.23-0.56%), acids (0.11-0.42%), alkyls (0.01-0.06%), alkene (0.02-0.04%), alkanes (0.01-0.03%), and lactones (0.00-0.12%). The most abundant compounds identified were isopulegol, pyranone, *cis*-4-methoxy, 1-penten-3-one, and spathulenol, though their concentrations fluctuated depending on the extraction parameters. Variations in temperature and pressure had a notable effect on the distribution of these compounds in terms of peak area percentages. Specifically, conditions of 50°C and 250 bars

exhibited the highest concentration of monoterpenes and the lowest levels of sesquiterpenes. Conversely, parameters of 40°C and 300 bars led to the highest levels of sesquiterpenes and the lowest levels of aldehydes.

The differences in monoterpenes and sesquiterpenes were due to the effects of pressure and temperature on their solubility in SC-CO_2. At 50°C and 250 bars, the temperature enhances the solubility of more volatile monoterpenes, while the moderate pressure maintains a high extraction efficiency (Jokić et al., 2022). In contrast, at 40°C and 300 bars, the lower temperature and greater pressure enhance the extraction of less volatile sesquiterpenes by increasing their solubility, while lowering the extraction of lighter molecules such as aldehydes (Pourmortazavi & Hajimirsadeghi, 2007). However, the overall findings indicated that *L. album* extract obtained at 50°C and 250 bars displayed the highest relative proportions of bioactive compounds.

4.2. The antifungal activity of plant extracts against *Fusarium* spp. (P3-P5)

The antifungal potential of *L. album* flower extracts was evaluated both *in vitro* and *in vivo*. The *in vitro* study was conducted on PDA medium in Petri dishes. For the *in vivo* experiments, wheat seeds were cultivated in pots under controlled growth chamber conditions, treated wheat kernels were incubated in laboratory conditions, and field trials were conducted to have a comprehensive overview of *L. album* flower extract against *Fusarium* pathogens. In all experiments, ergosterol and mycotoxins were analyzed because they are key indicators of fungal activity and pathogenicity. ERG is a vital structural and functional component in a fungal cell membrane that plays a crucial role in maintaining stability, fluidity, and integrity (Ferreira et al., 2018). It regulates membrane-bound enzyme activities, nutrient uptake, and cell signaling processes (Charcosset & Chauvet, 2001; Chen et al., 2018; Jafarzadeh et al., 2022) and serves as a biomarker for fungal biomass. It was analyzed by HPLC-PDA which was selected because it offers precise separation and accurate quantification of ergosterol. Mycotoxins, on the other hand, are toxic secondary metabolites produced by *Fusarium* spp that pose significant health risks to humans and animals and are believed to play a critical role in pathogenesis, including germination rates, seedling growth, pathogen aggressiveness, and overall disease severity (Morimura et al., 2020; Winter et al., 2019). UHPLC-HESI-MS/MS method was selected to analyze mycotoxins due to its high sensitivity, accuracy, and ability to simultaneously detect and quantify multiple mycotoxins in complex matrices (Tolosa et al., 2017). As a result, by analyzing both ERG and mycotoxins,

we were able to comprehensively evaluate the antifungal efficacy of *L. album* flower extract, determining not only its ability to inhibit fungal growth but also its potential to reduce mycotoxin biosynthesis.

4.2.1. In vitro experiment on PDA medium (P3)

After determining the optimal extraction conditions for *L. album* flowers based on antioxidant activity and chemical composition, the study proceeded to investigate the antifungal effects of the extract. The initial experiment was conducted *in vitro* to get a basic understanding of the extract's efficacy against *Fusarium* spp. specifically, *F. proliferatum* (PEA 1) and *F. culmorum* (KF 846) which were selected due to their significant impact on cereals in Europe, particularly Poland (Mielniczuk & Skwaryło-Bednarz, 2020; Okorski et al., 2022). *Fusarium* pathogens morphologically and molecularly identified were derived from the Institute of Plant Genetics at the Polish Academy of Sciences in Poznan, Poland.

The *in vitro* assay used the PDA medium because it is a nutrient-rich medium that supports the growth of *Fusarium* species and enables controlled conditions and accurate evaluation of the antifungal effects of *L. album* extracts. The poisoned food technique (Abhishek et al., 2015; Gakuubi et al., 2017) was used to determine the fungicidal efficiency of *L. album* flower extracts at four different concentrations (2.5, 5, 7.5, and 10%) where these extracts were mixed into PDA medium at 45°C, and mycelial discs from each *Fusarium* spp was placed in the center of the Petri dishes. The goal was to determine the lowest concentration of *L. album* flower extract that could prevent the growth of *F. proliferatum* and *F. culmorum* in a 10-day incubation period. The inclusion of both fungus species allowed for the investigation of potential susceptibility differences.

A significant inhibitory effect of *L. album* flower extracts on both *Fusarium* spp. compared to the control group (PDA without extract) was observed. As depicted in Figure 2, the observations showed the inhibitory effects of various concentrations (2.5, 5, 7.5, and 10%) of *L. album* flower extracts on the growth of *F. culmorum* and *F. proliferatum*. For *F. culmorum*, a noticeable trend in growth inhibition with increasing concentrations of *L. album* extract was observed. At lower concentrations (2.5 and 5%), there was a slight inhibitory effect on fungal growth, with a gradual increase in inhibition observed as the concentration of the extract increased to 7.5 and 10%. This suggests a dose-dependent relationship, where higher concentrations of *L. album* extract resulted in more significant inhibition of *F. culmorum* growth.

Similarly, for *F. proliferatum*, the results indicated a significant inhibitory effect of *L. album* extract across all concentrations tested. Even at the lowest concentration (2.5%), there was a noticeable reduction in fungal growth compared to the control, with further inhibition observed at higher concentrations. This suggests that *L. album* extract possesses potent antifungal properties against *F. proliferatum*, with the inhibitory effect being more pronounced at higher concentrations.

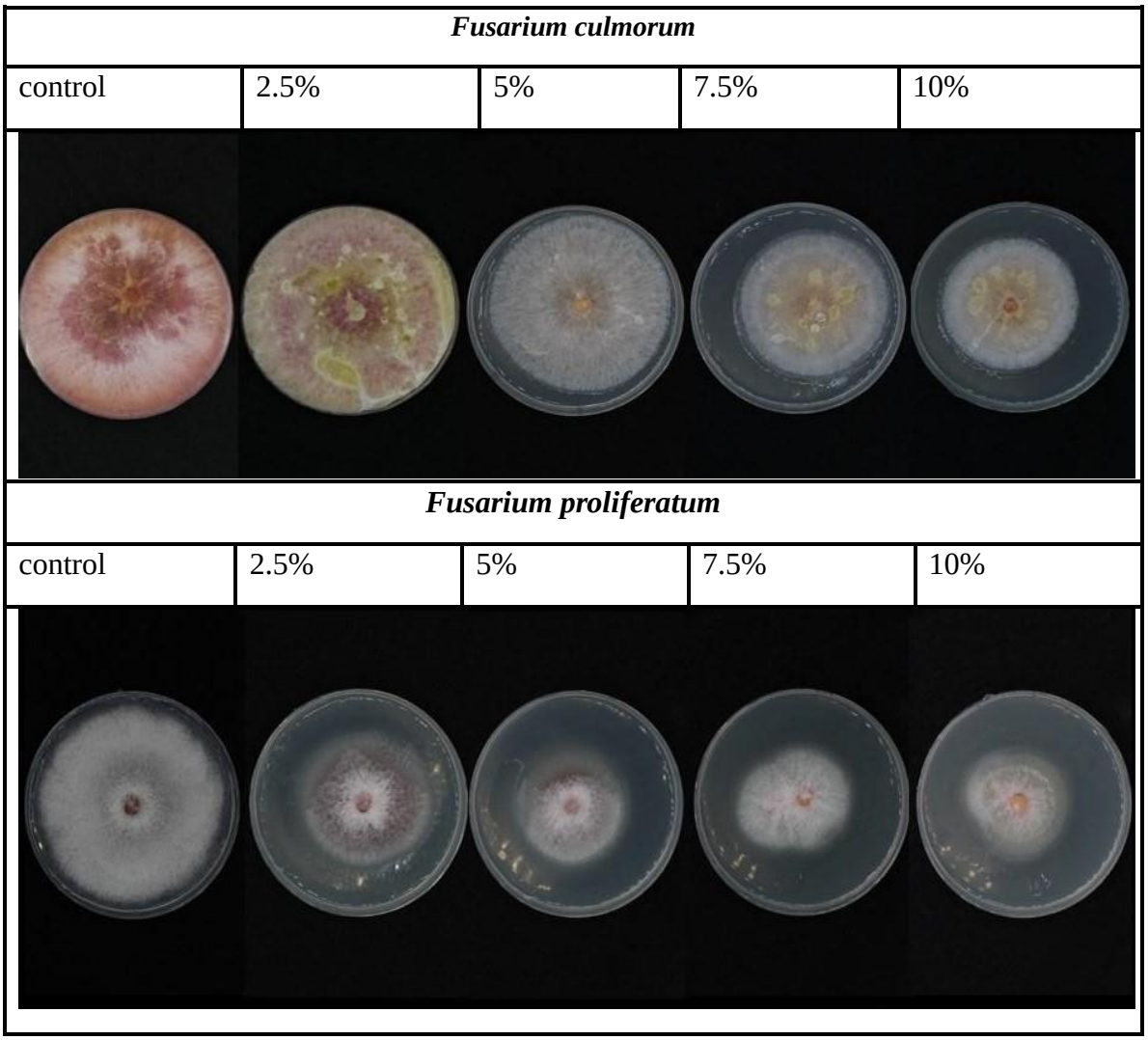


Figure 2. The inhibitory effects of *L. album* flower extract at different concentration levels (2.5-10%) on mycelia growth of *F. culmorum* and *F. proliferatum* in the PDA medium after a 10-day incubation period

Furthermore, Figure 3 demonstrates the inhibitory effect of *L. album* flower extracts on *F. culmorum* and *F. proliferatum* over a 10-day incubation period. For *F. culmorum*, it was observed that the inhibitory effect of the extract concentrations (2.5, 5, 7.5, and 10%) varies with the duration of the incubation period. During the first few days of incubation,

particularly up to day 3, the inhibitory effect of the lower concentrations (2.5 and 5%) was not pronounced, with minimal inhibition observed. However, as the incubation period progressed, the inhibitory effect became more evident, especially for the higher concentrations (7.5 and 10%), reaching significant inhibition levels by day 10. A similar trend was observed for *F. proliferatum*; the inhibitory effect of the *L. album* flower extracts increased over the 10-day incubation period. Initially, all concentrations exhibit similar levels of inhibition during the early stages of incubation. However, by the sixth day of incubation, the inhibitory effects of the extract concentrations begin to manifest, with increasing inhibition observed for higher concentrations. By the last day of incubation, all concentrations showed a positive inhibitory effect compared to the control group. Interestingly, the difference in growth inhibition between concentrations was less pronounced compared to *F. culmorum*, suggesting that even lower concentrations of *L. album* extract effectively inhibited the growth of *F. proliferatum*.

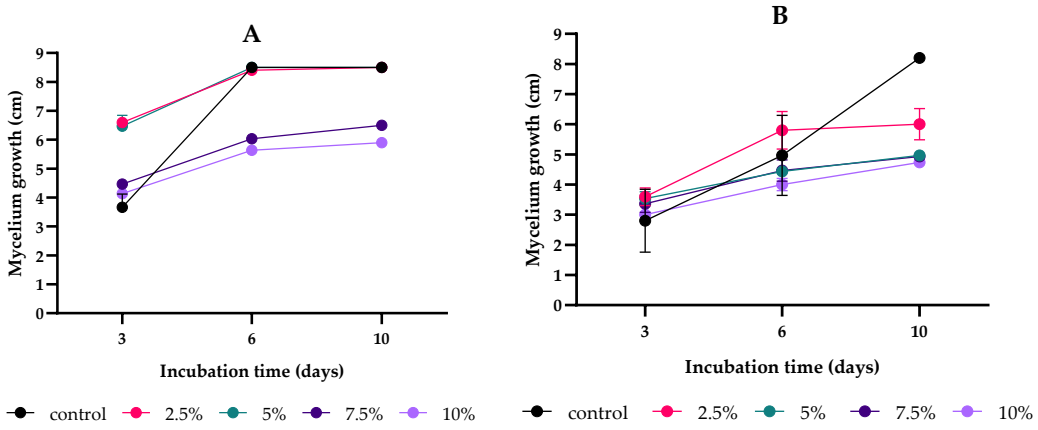


Figure 3. Growth response of *Fusarium culmorum (A)* and *Fusarium proliferatum* (B) to *L. album* extract concentrations over 10 days

Notes: The results are averages of three repetitions ± standard deviation.

The different responses of *F. culmorum* and *F. proliferatum* by applying *L. album* were due to fundamental variations in these species' genetic composition, metabolic pathways, and physiological mechanisms. The inhibitory effect on *F. culmorum* depended on the concentration of the *L. album* extract, indicating that higher concentrations of the extract may have a stronger impact on the growth and development of this fungi. On the other hand, the lack of a similar trend in *F. proliferatum* could indicate a different threshold of susceptibility to the extract's bioactive compounds, resulting in a less pronounced response to increasing concentrations. These observations are consistent with previous studies highlighting the

diverse effects of natural plant extracts on the proliferation of different fungi. For instance, Reyes-Vaquero et al. (2021) demonstrated differential inhibition by the extract of *Ruta graveolens* against F. *proliferatum* and *F. oxysporum* (Reyes-Vaquero et al., 2021); similarly, Kursa et al. (2022) reported distinct inhibitory effects of *Salvia officinalis* L. against three *Fusarium* strains (Kursa et al., 2022). In addition, Ngegba et al. (2018) showed that *Chromolaena odorata, Azadirachta indica,* and *Tithonia diversifolia* inhibited *F. oxysporum* differently at the same concentration (Ngegba et al., 2018). This indicates that the efficacy of plant extracts in combating *Fusarium* pathogens is influenced by the distinct characteristics of each plant material, the concentration used, and its interaction with the pathogen.

ERG content was significantly reduced in both tested *Fusarium* spp. in a concentration-dependent manner. This decrease was most likely attributed to the increasing antifungal activity of the *L. album* extract at higher concentrations. As the concentration of the *L. album* flower extract increases, it more effectively interrupts ergosterol production or destroys the fungal cell membrane, leading to reduced ergosterol levels. This interruption in ergosterol synthesis ultimately inhibited fungal growth and vitality which is reflected in the observed decrease in ERG content between the extracts treated samples and the control (Table 2).

Table 2. Effects of *L. album* extracts (2.5, 5, 7.5, and 10%) on ERG content [µg/g] and its reduction [%] after 10 days of incubation at 25°C on a PDA medium inoculated with *Fusarium* species

Extract concentration [%]	**ERG concentration [µg/g] and percentage of reduction [%]**			
	F. culmorum		***F. proliferatum***	
	[µg/g]	**[%]**	**[µg/g]**	**[%]**
Control (without extracts)	5036.19 ± 1178.93^{c}	-	20234.01 ± 1484.40^{d}	-
2.5	3818.97 ± 829.56^{bc}	24.17^{c}	7778.33 ± 227.47^{c}	61.56^{c}
5	1864.53 ± 706.63^{ab}	62.98^{ab}	4278.58 ± 281.07^{b}	78.85^{b}
7.5	1016.00 ± 200.36^{a}	79.83^{ab}	1549.54 ± 157.36^{a}	92.34^{a}
10	560.59 ± 140.09^{a}	88.87^{a}	1381.55 ± 191.88^{a}	93.17^{a}

Notes: All values are means of three replicates ± standard deviation. The superscripts of different letters in rows are significantly different (Tukey's HSD test, significant at $p < 0.01$).

At the lowest tested concentration (2.5%) the extract inhibited the growth of *Fusarium*, which is confirmed by the ERG content being approximately 24.17% lower for *F. culmorum* and 61.56% lower for F. *proliferatum*. With higher extract concentrations (5, 7.5, and 10%),

the reduction in ERG became more pronounced, with the 10% concentration exhibiting the most significant inhibition of *Fusarium* growth, with ERG reduction in the average of 88.87% for *F. culmorum* and 93.17% for *F. proliferatum* compared to the control. These results align with previous reports on the antifungal effects of plant extracts and essential oils obtained from medicinal plants (Bodoira et al., 2020; Perczak et al., 2019; Somai et al., 2021). Bodoira et al. (2020) reported that the extracts obtained from agro-industrial by-products of peanut, sesame, and pistachio reduced ergosterol content by 25, 66, and 33%, respectively, compared to the control. Similarly, another study reported a significant decrease in ERG production in the presence of plant extract compared to the untreated control (Somai et al., 2021).

In addition, all tested concentrations of *L. album* flower extracts demonstrated a reduction in mycotoxin biosynthesis compared to the control (PDA without extracts). For *F. culmorum*, the biosynthesized mycotoxins were reduced as follows: DON (44.39–96.41%), 3- and 15-AcDON (61.64–96.94%), ZEN (49.38–89.71%), ZEN-14S (52.33–92.61%), β-ZOL (55.57–99.86%), α-ZOL (68.42–100%), and FUS-X (55.57–99.51%) while for the *F. proliferatum*, the mycotoxin biosynthesis was similarly reduced in the average range as follows: BEA (48.50– 86.76), FB_1 (39.35–87%) FB_2 (31.48–81%), and FB_3 (51.51–90%) with the extent of suppression varying based on extract concentration, *Fusarium* spp., and type of mycotoxin. The concentration of 10% was the most effective in reducing mycotoxin biosynthesis in both species. The studied *Fusarium* species produced different mycotoxin classes at different levels. *F. culmorum* exhibited various mycotoxins, including type B trichothecenes (DON, its derivatives–3- and 15-AcDON and FUS-X), as well as ZEN and its derivatives (ZEN-14S, β-ZOL, and α-ZOL) where the highest content of ZEN-14S was observed compared to other mycotoxins identified *in vitro* and *in vivo*. This mycotoxin (ZEN-14S) is recognized as an emerging mycotoxin due to its recent identification, and ongoing research continues to uncover its prevalence, toxicological properties, and potential impact on health. Veršilovskis et al., (2019) noted that ZEN-14S is approximately 60 times more estrogenic than ZEN and can be readily hydrolyzed to ZEN in the gastrointestinal tract, thereby increasing exposure to ZEN (Veršilovskis et al., 2019) and also a high ZEN-14S/ZEN ratio in the malted wheat suggests that *Fusarium* can convert ZEN into a phase II metabolite through sulfation reactions (Ksieniewicz-Woźniak et al., 2021).

On another hand, *F. proliferatum* synthesized different mycotoxins, including FBs (FB_1, FB_2, FB_3) and beauvercin (BEA), the most predominated mycotoxin. BEA is a toxic mycotoxin that has been recognized as an inhibitor of cholesterol acyltransferase and has demonstrated its toxicity toward various human cell lines.in addition, it has been shown to induce apoptosis and cause DNA fragmentation (Hasuda, A. L., & Bracarense, A. P. F. R. L., 2024). Different studies have reported its highest prevalence in grains and wheat-based products such as pasta, breakfast cereals, biscuits, and infant formulas, with incidence rates ranging from 40 to 90% (Deng et al., 2018). However, primary food regulatory bodies like the US Food and Drug Administration and the European Food Safety Authority have not yet established specific regulations or guidelines for permissible levels of BEA and emerging mycotoxins (Svingen et al., 2017) due to the lack of data on the *in vivo* toxicity of these mycotoxins.

Despite the prominence of different mycotoxins, *L. album* flower extract demonstrated efficacy against all produced mycotoxins. This highlights the importance of identifying and characterizing a diverse set of mycotoxins to ensure an accurate assessment of mycotoxin content and mitigate potential health risks to consumers (Marc, 2022). Furthermore, our study showed that *L. album* flower extracts can inhibit the biosynthesis of well-known mycotoxins and their modified forms and derivatives. This is consistent with previous research that reported the anti-mycotoxigenic effect of plant extracts on various mycotoxins (Barral et al., 2020; Heidtmann-Bemvenuti et al., 2016; Velluti et al., 2003).

4.2.2. Impact of extracts on seed germination and seedling growth under controlled growth conditions (P4)

As a continuation of the research, the effects of *L. album* flower extracts at concentrations of 5 and 10% were analyzed to assess their impact on seed germination and seedling growth under a controlled growth chamber environment of natural photoperiod of 16 hours of light and 8 hours of darkness at a temperature of 23±4°C and with 45% humidity. The selection of these concentrations was based on their demonstrated inhibitory potential against *Fusarium* in the *in vitro* experiment. Testing these concentrations allowed for the determination of efficacy and potential phytotoxicity of the extracts where the 5% concentration was used as a reference point to measure moderate effects, while the 10% concentration was used to analyze any additional benefits or adverse effects at higher doses. Initially, the wheat seeds were placed on glass plates and left to incubate for 7 days to assess

the early impact of the extracts on germination and seedling vigor. This assessment is crucial because, while the extract may have antifungal properties, it must also be safe for the plants themselves. The seedlings were then transferred to pots in a controlled chamber, and after 10 days of further growth, they were artificially inoculated with *F. culmorum*. Following an additional 21 days of growth after inoculation, the effects of the *L. album* flower extracts on seedling development were assessed by measuring roots and leaves heights and weights. These plant parts were further collected and analyzed for ergosterol and mycotoxin content. This approach allowed for a comprehensive evaluation of how *L. album* flower extracts influence seedling development and their potential protection against *F. culmorum* in wheat seedlings.

The phytotoxic effects of *L. album* flower extract on wheat seeds were determined on seed germination and seedling growth where findings showed a dose-dependent reduction of seed germination. While the control group exhibited a 100% germination rate, the extract-treated samples showed significant reductions, with germination rates of 65% for the 5% extract and 45% for the 10% extract. The extracts also had a detrimental impact on the seedling length vigor index which declined from 2860 in the control to 669.50 at 5% and then to 303.86 at 10%. Similarly, the seedling weight vigor index decreased from 3.92 in the control group to 2.83 at 5% and 1.84 at 10%. Additionally, the extract concentration had a substantial effect on wheat seedling shoot and root lengths (Table 3 and Figure 4), with no significant difference in dry weight. An increase in *L. album* concentration led to a noticeable decrease in both shoot and root length.

Table 3. Phytotoxic effect of *L. album* flower extracts on germination characteristics of wheat seeds

Extract concentration [%]	seed germination [%]	shoot length [cm]	root length [cm]	dry weight [mg]
Control (without extracts)	100^{a}	$12.88^{a} \pm 0.77$	$15.73^{a} \pm 1.00$	$39.15^{a} \pm 5.14$
5	65^{b}	$6.98^{b} \pm 0.19$	$3.33^{b} \pm 0.77$	$43.46^{a} \pm 4.40$
10	45^{c}	$4.55^{c} \pm 0.09$	$2.20^{b} \pm 0.44$	$40.83^{a} \pm 3.90$

Notes: values with different letters are statistically different at $p < 0.05$ based on the post hoc Duncan's multiple range test.

At a 10% *L. album* concentration, the germinated seeds exhibited shoot and root lengths of 4.55 cm and 2.20 cm, respectively while, at a 5% *L. album* concentration, the shoot and

root lengths were 6.975 cm and 3.32 cm, respectively, both shorter than the untreated control, which displayed shoot and root lengths of 12.88 cm and 15.73 cm, respectively.

These findings, although unexpected, align with previous studies reporting the phytotoxic effects of various plant extracts on wheat germination (Hamouda et al., 2022; Dai et al., 2022; Joshi and Joshi, 2016). The observed phytotoxic effect could be attributed to the concentration of the extract, its chemical composition, or interactions with seed physiology (Dai et al., 2022; Hamouda et al., 2022; Rys et al., 2022; Scavo et al., 2022) as the increase of *L. album* concentration, resulted in the high decrease of germination rate and seedling stunting. Therefore, it is crucial to analyze the phytotoxic effect of any plant extract before considering or applying it as an antifungal, as diverse effects may occur to the plant intended to protect.

Figure 4. The effects of different concentrations of *L. album* flower extract (5 and 10%) compared to the untreated control (treated with water) on seedlings' shoot and root length after 7 days of incubation

Comparative analysis of *L. album* flower extracts on the physiological growth parameters of wheat seedlings, both in the presence and absence of *F. culmorum* infection, was conducted to determine if the extract could mitigate the harmful effects of *F. culmorum* and possibly promote healthier growth in infected plants. *F. culmorum* is known to severely impair the growth and development of wheat by causing disease and reducing vital growth parameters like root length, and overall biomass (Saad et al., 2023). By comparing these parameters in wheat seedlings treated with *L. album* flower extract, both with and without

fungal infection, the results showed that *F. culmorum* infection and *L. album* flower extract had significant effects on the physiological growth of wheat seedlings, specifically roots (Table 4). In pathogen-inoculated seedlings treated with 5% and 10% of *L. album* flower extracts, root weight slightly decreased by approximately (−0.3 g) compared to the infected control group where no extract was used. In contrast, non-infected seedlings treated with the extract experienced a significant reduction in root weight (−1.8 g) compared to the non-treated control. The slight decrease in root weight observed in seedlings exposed to pathogens and treated with a 5% or 10% concentration of *L. album* extract, in comparison to the infected control group, suggests that the extract might have partially mitigated the detrimental impacts of *F. culmorum*, but not entirely. On the other hand, the noticeable decrease in root weight observed in healthy seedlings treated with the extract suggests that, without any infection, the extract may have a slightly harmful effect on plants, impacting root growth as was mentioned earlier.

Table 4. Comparative effect of *L. album* extracts on the roots of infected and non-infected wheat seedlings

	Variants	root weight	root length
		[g]	[cm]
non-infected	without extract	$2.95^{ab} \pm 1.15$	$39.25^{b} \pm 6.85$
	5 % extract	$1.04^{b} \pm 0.48$	$37.50^{b} \pm 8.74$
	10 % extract	$1.32^{b} \pm 0.13$	$56.25^{a} \pm 16.78$
F .culmorum	without extract	$3.24^{a} \pm 1.16$	$48.5^{ab} \pm 6.25$
	5 % extract	$2.94^{ab} \pm 0.66$	$45.75^{ab} \pm 8.22$
	10 % extract	$2.96^{ab} \pm 1.16$	$36.25^{b} \pm 6.95$

Notes: All values are means of four replicates with standard deviation. The values assigned with different letters are statistically different ($p < 0.05$).

However, different results were observed in the root lengths of the samples where 10% of the extract was used, non-infected seedlings with 10% extract had the longest roots while the infected seedlings with 10% showed the shortest root length where the possible detrimental effect of *Fusarium* infection and protective effect of *L. album* flower extracts could be noted. *F. culmorum* infection can impact plants across different developmental stages, leading to seedling blight and root rot, hindering seedling emergence and overall plant development (Pastuszak et al., 2021) previous studies reported that the early infection

reduced wheat seedling growth as *F. culmorum* can effectively penetrate seedling roots, migrate from hypocotyl to the upper stem internodes and leaves, colonize the host's tissue and cells, block the vascular bundles, disturb nutritional supply and metabolic processes (Kthiri et al., 2021). However, on the other hand, the reduction in root length observed may indicate a positive influence of *L. album* flower extract in counteracting *Fusarium*, where shorter roots could potentially serve as a defense response, limiting *Fusarium* invasion and enhancing plant resistance. In addition, the inhibitory action of *L. album* flower extract was clearly observed. Infected seedlings that had not been treated exhibited signs of infection. However, wheat seedlings treated with *L. album* showed no symptoms (Figure 5). Therefore, these varied responses found in this study illustrate the complex interaction between the extract, the plant, and the pathogen. The extract can exhibit defensive capabilities in the presence of plant pathogens, but it may hinder growth in uninfected plants.

Figure 5. Visual evidence of the inhibitory effect of *L. album* extract on fungal infection in wheat seedlings

To further assess the extent of *Fusarium* infections in wheat seedlings, the fungal biomarker ERG was quantified in both artificially inoculated seedlings by *F. culmorum* and non-inoculated seedlings. This analysis provided insight into the antifungal efficacy of *L. album* flower extracts. The results showed the presence of ERG, both in non-infected and infected seedlings Figure 6.

In the non-infected seedlings, the control group exhibited the highest ERG level in leaves and roots, however, ERG levels were significantly lower in seedlings treated *with L. album* extracts, suggesting a reduction in fungal colonization, regardless of whether the tissue was from roots or leaves. The presence of ERG in those samples was attributed to the endophytic fungi ― fungi that reside within plant tissues for part or all of their life cycle,

forming a mutually beneficial symbiotic relationship with the host plant without causing harm or inducing disease (Wen et al., 2022).

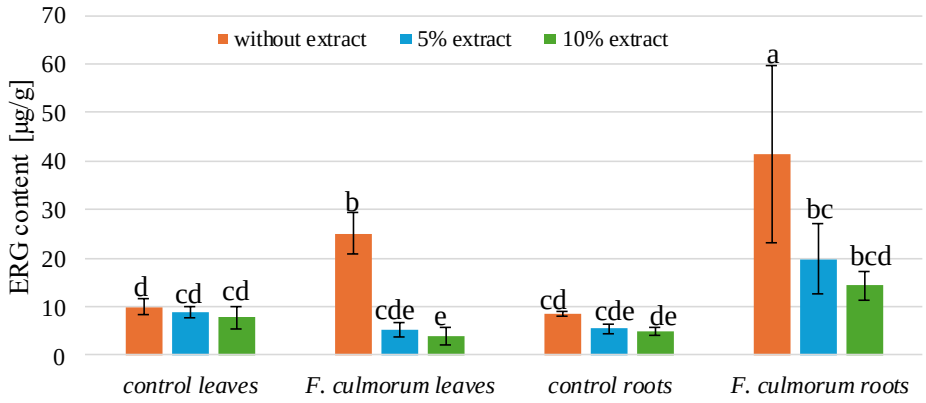


Figure 6. Ergosterol concentrations [µg/g] in roots and leaves of wheat seedlings inoculated with and without *F. culmorum* in the control and treated samples with *L. album* flower extracts

Notes: All values are means of four replicates; error bars represent the standard deviation. The values assigned with the superscripts of different letters are significantly different at $p < 0.05$ based on post hoc Duncan's multiple range test.

In the infected seedlings, ERG content was consistently lower in extract-treated plants compared to the untreated control, further confirming the antifungal activity of *L. album* extracts. A significant variation in ERG concentration was observed between roots and leaves, with roots containing higher levels of ERG. This is likely due to the infection initiating in the root system and not fully spreading to the above-ground tissues. Additionally, the antifungal effect of the extracts was dose-dependent. Treatment with a 10% extract concentration resulted in an average ERG content of 14.30 µg/g in roots and 3.87 µg/g in leaves, while the 5% extract treatment yielded higher ERG concentrations of 19.85 µg/g in roots and 5.19 µg/g in leaves.

To evaluate the influence of *L. album* flower extracts on mycotoxin content within the roots and leaves of seedlings artificially infected with *F. culmorum*, compared to non-infected groups four commonly synthesized mycotoxins of *F. culmorum* - DON, 3- and 15-AcDON, ZEN, and ZEN-14S were identified and quantified. Table 5 indicates that treatment with *L. album* flower extracts resulted in a noticeable reduction in mycotoxin accumulation within the seedlings compared to the control after 21 days of inoculation.

Table 5. Impact of *L. album* on the mycotoxin biosynthesis [ng/g] in *Fusarium*-infected wheat seedlings and their reductions [%]

Mycotoxins	Treatments	Seedling parts	
		Leaves	**Roots**
DON	Control	nd^{b}	18.90^{a} ± 13.37
	5%	nd^{b}	nd^{b}
	10%	nd^{b}	nd^{b}
3- and 15-AcDON	Control	nd^{b}	30.31^{a} ± 12.10
	5%	nd^{b}	nd^{b}
	10%	nd^{b}	nd^{b}
ZEN	Control	31.30^{b} ± 6.70	56.01^{a} ± 8.48
	5%	nd^{d}	10.00^{c}± 2.48 (82.14)
	10%	nd^{d}	7.28^{c} ± 1.73 (87.41)
ZEN-14S	Control	46.26^{a} ± 4.66	51.68^{a} ± 12.33
	5%	nd^{c}	12.90^{b}± 3.59 (75.03)
	10%	nd^{c}	10.87^{b} ± 1.05 (78.97)

Notes: All the values are the mean of four replicates ± standard deviation. Values with different letters are statistically different at $p < 0.05$ based on the post hoc Duncan's multiple range test. The values in parentheses are the degree of mycotoxin reduction in %. DON (deoxynivalenol), 3- and 15-AcDON (3- and 15-acetyl deoxynivalenol), ZEN (zearalenone), ZEN-14S (zearalenone-14-sulfate); nd - not detected.

The highest concentration of mycotoxins was in the roots compared to the leaves, with ZEN and ZEN-14S exhibiting higher levels than DON and 3- and 15-AcDON; this could be attributed to the fact that the roots were in direct contact with the soil where *F. culmorum* were primarily infected, facilitating more significant fungal colonization and mycotoxin accumulation.

Additionally, roots serve as initial infection sites, allowing the fungus to proliferate and produce mycotoxins more abundantly before systemic spread to other plant parts like the leaves. Applying *L. album* extract in the infected seedlings resulted in a complete inhibition (100%) of DON and 3- and 15-AcDON in the roots. However, ZEN was entirely inhibited in the leaves, but its concentration in the roots decreased to 82.14 and 87.41% for *L. album* extract concentrations of 5 and 10%, respectively. Similarly, ZEN-14S was inhibited entirely in leaves, while in the roots, its concentration decreased to 75.03 and 78.97% for *L. album* extract concentrations of 5 and 10%, respectively. Furthermore, consistent with expectations, non-infected wheat seedling parts (roots and leaves) showed no presence of mycotoxins.

Overall, these findings demonstrate a significant impact of *L. album* on mycotoxin biosynthesis in the infected seedlings, resulting in a notable decrease in mycotoxin concentrations compared to the control, irrespective of the extract concentration, as differences were statistically insignificant for all produced mycotoxins.

4.2.3. In vivo experiment – wheat kernel pre-treatments and wheat cultivation under field conditions (P5)

After the promising results on the antifungal effects of *L. album* flower extracts against *Fusarium* pathogens *in vitro* and controlled environment. Further studies were continued to evaluate the practical feasibility and efficacy of *L. album* flower extracts on wheat kernels incubated in laboratory conditions at room temperature and wheat cultivation in the field when exposed to *Fusarium* infection. In the laboratory experiment, three distinct pre-treatment methods (non-autoclaved, autoclaved, and microwaved) coupled with *L. album* treatments were evaluated on wheat kernels to assess how these different preparation methods influenced the antifungal efficacy of the extracts. These approaches were crucial in determining whether the integrity and activity of the bioactive compounds in *L. album* flower were maintained or enhanced through these processes, ultimately affecting their ability to inhibit fungal growth on wheat kernels.

Table 6 shows significant differences in ERG and mycotoxin content among the wheat kernel pre-treatment groups (non-autoclaved, autoclaved, and microwave) in response to *F. culmorum* infection. The sterilization methods and the application of *L. album* flower extract influenced these differences as was shown by the ANOVA statistical analysis, with post hoc Tukey test with $p < 0.05$.

In the autoclaved control group, ERG content reached the highest levels (501.25 μg/g), indicating robust fungal growth due to the absence of microbial competition, as autoclaving sterilizes the grains, allowing *F. culmorum* to proliferate without interference. Similarly, this group exhibited the highest concentrations of mycotoxins, including ZEN-14S (56.78 μg/g and ZEN (36.07 μg/g), highlighting substantial toxin biosynthesis in the absence of competing microorganisms. However, the application of *L. album* extract significantly reduced ERG content to 111.75 μg/g - over five times lower than the autoclaved control - demonstrating the extract's strong antifungal properties in a sterile environment. Similarly, the extract significantly reduced the biosynthesis of all analyzed mycotoxins (DON, 3-/15-AcDON, ZEN, ZEN-14S) compared to the control.

Table 6. Comparative analysis of wheat kernel sterilization methods and the efficacy of *L. album* extract against *F. culmorum* infection (data not published)

Variants		ERG	DON	3-/15-AcDON	ZEN	ZEN-14S
		[µg/g]				
non-autoclaved	*F. c.*	$168.21^{b} \pm 82.41$	$1.43^{c} \pm 0.67$	$3.15^{b} \pm 1.86$	$6.14^{c} \pm 4.05$	$17.14^{b} \pm 7.67$
	F. c. + extract	$142.99^{b} \pm 36.50$	$1.23^{c} \pm 0.22$	$2.23^{b} \pm 1.00$	$4.77^{c} \pm 2.29$	$15.64^{b} \pm 7.46$
autoclaved	*F. c.*	$501.25^{a} \pm 197.25$	$17.41^{a} \pm 4.31$	$15.68^{a} \pm 6.82$	$36.07^{a} \pm 7.56$	$56.78^{a} \pm 18.00$
	F. c. + extract	$111.75^{b} \pm 62.84$	$5.15^{bc} \pm 1.50$	$4.83^{ab} \pm 2.95$	$15.45^{bc} \pm 4.18$	$26.01^{b} \pm 4.99$
microwaved	*F. c.*	$227.53^{b} \pm 41.96$	$11.57^{ab} \pm 3.08$	$8.32^{ab} \pm 3.02$	$25.48^{ab} \pm 4.59$	$18.18^{b} \pm 7.48$
	F. c. + extract	$196.79^{b} \pm 31.44$	$9.17^{ab} \pm 2.24$	$7.90^{ab} \pm 1.94$	$24.81^{ab} \pm 9.26$	$18.62^{b} \pm 6.40$

Notes: All values are means of three replicates ± standard deviation. The superscripts of different letters are significantly different at $p<0.05$ based on the Post hoc Tukey HSD test. Variants description: *F.c*: *Fusarium culmorum* (control group without extract for each treatment)

In the microwave pre-treated group, both the extract-treated and control samples showed lower ERG content compared to the autoclaved control, with microwave control showing 227.53 µg/g and the extract-treated group having a reduced level of 196.79 µg/g. Although the reduction in ERG was less pronounced than in the autoclaved samples, it still indicates some antifungal efficacy of *L. album* extract. Similarly, mycotoxin levels, including DON and ZEN, were lower in extract-treated samples compared to the control, though the reduction was not as significant as in the autoclaved group. This suggests that the sterilization method influences both fungal activity and the efficacy of the extract, with microwave treatment possibly inactivating some microorganisms, but still allowing some competing organisms to affect *Fusarium* growth and reduce mycotoxin biosynthesis.

In contrast, the non-autoclaved samples (extract-treated and control groups), had the lowest ERG levels (142.99 µg/g and 168.21 µg/g, respectively), and mycotoxins specifically DON and ZEN, with extract-treated samples showing 1.23 µg/g for DON and 4.77 µg/g for ZEN, which can be attributed to the presence of other microorganisms in these samples, which likely competed with *F. culmorum* for resources, naturally inhibiting its growth.

Additionally, the impact of the extract was less prominent, as the non-sterile conditions already suppressed fungal proliferation. These findings show the necessity of sterilization in impacting fungal development, as well as the efficacy of *L. album* flower extract in lowering ERG and mycotoxin levels, especially under sterile conditions.

At last, the field experiment was carried out at the experimental station of the Institute of Plant Protection, National Research Institute in Winna Góra, Poland, during the 2022/2023 growing season to determine the practical efficacy of *L. album* flower extract in spray treatment. The reason for selecting this technique was because the method of introducing the extract into the soil by watering as tested in the controlled chamber experiment was not entirely effective. Therefore, spraying was used in field conditions to potentially enhance the effectiveness of the treatment. Two winter wheat cultivars with different susceptibility to *Fusarium* were selected for the field experiment: Julius a susceptible cultivar, and Arkadia a more resistant cultivar. During the BBCH 63 phase (flowering stage), *F. culmorum* spore suspensions were inoculated, followed by the application of *L. album* flower extracts This phase of wheat growing was intentionally targeted because this is one of the most essential stages for *Fusarium* infections, particularly those that cause FHB (György et al., 2020). This stage is particularly susceptible to infection because open flowers provide entry points for the pathogen, which can have a significant influence on wheat yield and quality. Therefore, the study used *L. album* extracts at this vulnerable stage to determine the treatment's efficacy when the crop was at the highest risk of infection. This timing also allowed for a more realistic assessment of *L. album*'s potential to protect wheat against *F. culmorum* in conditions that were similar to natural infection. The wheat cultivation was monitored and at the maturity stage, wheat grains were harvested and analyzed for ergosterol and mycotoxin levels.

As depicted in Figure 7, the application of *L. album* flower extracts against artificially inoculated *F. culmorum* in winter wheat cultivars resulted in a distinct decrease in ERG content for both cultivars. In the case of the Arkadia cultivar, the control group exhibited ERG levels ranging from 18.64 to 35.92 µg/g, with an average of 26.07 µg/g. Conversely, the extract-treated group demonstrated a significant decrease in ERG content, ranging from 5.63 to 13.01 µg/g, with an average of 8.91 µg/g. Similarly, in the Julius cultivar, the control group displayed ERG content ranging from 46.61 to 116.17 µg/g, averaging 70.02 µg/g. In contrast, the extract-treated group exhibited a noticeable reduction in ERG content, ranging from 20.88

to 35.62 µg/g, with an average of 30.20 µg/g. Notably, the Arkadia cultivar demonstrated a more substantial reduction in ergosterol, marking a 65.82% decrease compared to the control group. In contrast, the Julius cultivar exhibited a 56.86% reduction in ERG relative to the control groups. These differential impacts of *L. album* extract on ergosterol reduction highlight the potential variations in cultivar response to fungal infection and the antifungal treatment. Arkadia proves its more resistant to *F. culmorum* compared to Julius, which exhibited greater susceptibility. These findings confirm the efficacy of *L. album* extracts in reducing ergosterol content in both wheat cultivars, underscoring their antifungal properties.

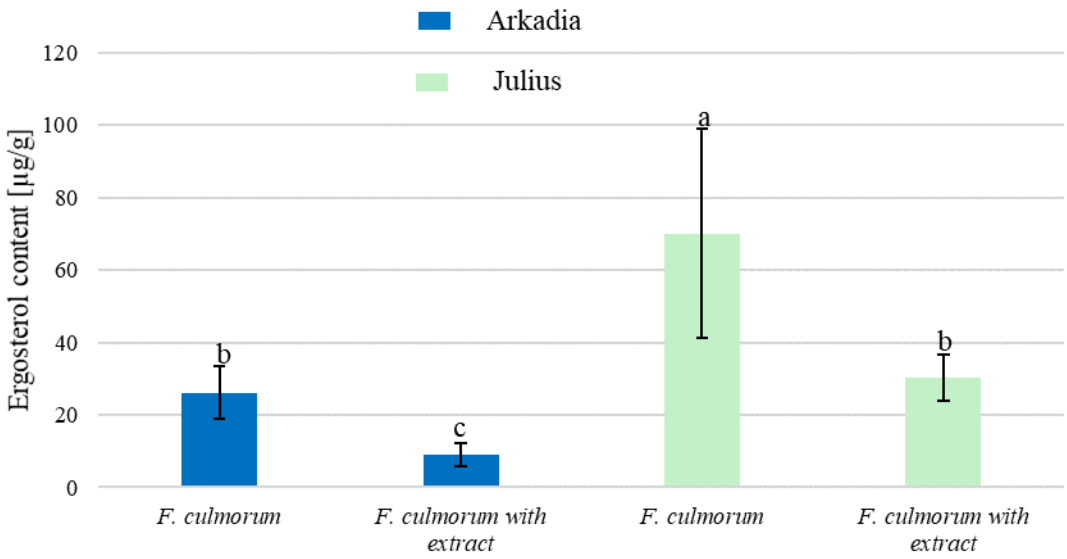
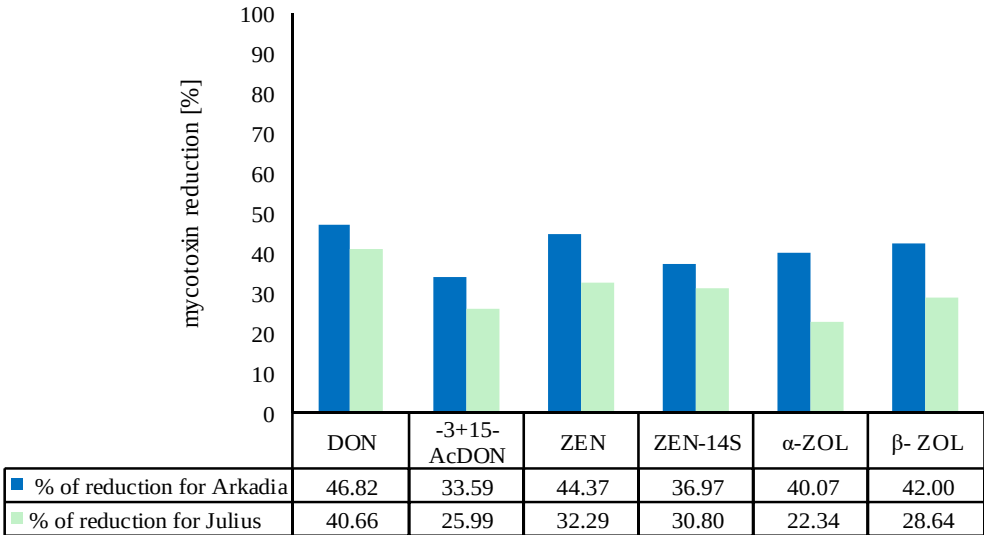


Figure 7. Effects of *L. album* extract on ERG content in Arkadia and Julius wheat cultivars inoculated with *F. culmorum*

Notes: All values are means of four replicates; error bars represent the standard deviation**.** Different letters correspond to significant differences ($p < 0.05$) between means based on post-hoc tests (Duncan test).

Furthermore, the effect of *L. album* flower extracts *on* mycotoxin biosynthesis was noted in the harvested grain, with a lower concentration of the produced mycotoxins: DON, 3- and 15-AcDON, ZEN, ZEN-14S, α-ZOL, and β-ZOL compared to the control groups in both cultivars Arkadia and Julius. Among the biosynthesized mycotoxins in both cultivars, ZEN-14S (285.71–668.97 µg/g) was the most prominent, while β-ZOL (1.09–2.94 µg/g) and α-ZOL (0.26–3.53 µg/g) were the least produced. However, the levels of mycotoxins were significantly different in both cultivars, with the Julius cultivar showing a higher accumulation of mycotoxins than the Arkardia due to their susceptibility to *Fusarium* infection and their inherent genetic and physiological differences. These findings align with

existing literature, reinforcing the significant varietal differences in susceptibility to *Fusarium* infection and mycotoxin accumulation in winter wheat cultivars (Jończyk et al., 2018; Sunic et al., 2021). In addition, the potential of *L. album* in reducing mycotoxins was observed (Figure 8) in both cultivars, with the Arkadia cultivar exhibiting a more substantial reduction in mycotoxin levels, ranging between 33.59 to 46.82%, compared to the Julius cultivar, where mycotoxins were reduced in the range from 22.34 to 40.66%. In both varieties, DON and ZEN were the most reduced mycotoxins.



	DON	-3+15-AcDON	ZEN	ZEN-14S	α-ZOL	β- ZOL
% of reduction for Arkadia	46.82	33.59	44.37	36.97	40.07	42.00
% of reduction for Julius	40.66	25.99	32.29	30.80	22.34	28.64

Figure 8. Comparative effect of *L. album* on mycotoxin reduction in winter wheat cultivars Arkadia and Julius

Notes: Data are expressed as % of mycotoxins reduction. Analyzed mycotoxins: deoxynivalenol (DON), 3- and 15-acetyl deoxynivalenol (3- and 15-AcDON), zearalenone (ZEN), zearalenone-14-sulfate (ZEN-14S) and beta-zearalenol (β-ZOL) and alpha-zearalenol (α-ZOL).

In summary, the key achievement of this study is the demonstration of *L. album* flower extracts as a promising natural antifungal agent for reducing *Fusarium* infection in wheat. The research successfully optimized the supercritical CO_2 extraction process to obtain bioactive-rich extracts, with notable antioxidant potential and antifungal efficacy against *Fusarium* species. *In vitro*, *L. album* flower extracts exhibited significant inhibitory effects against *F. culmorum* and *F. proliferatum* grown on PDA medium. *In vivo*, the antifungal potential of the extracts was further validated by reduced ERG and mycotoxin levels in wheat seedlings, wheat kernels, and field trials infected with *F. culmorum*. Additionally, the study highlights the importance of balancing antifungal efficacy with potential phytotoxic effects on crop growth. Overall, this study represents an important step toward developing eco-

friendly and natural alternatives to synthetic fungicides for managing *Fusarium* infections in wheat.

5. Conclusions

Biological control and the search for new natural antifungal agents are gaining more and more interest. The study's findings significantly address the challenge of managing *Fusarium* pathogens in agriculture. The study explored the potential of *L. album* flower extracts for controlling *Fusarium* species through a series of experiments. The extraction method using SC-CO_2 with methanol as a co-solvent yielded extracts abundant in bioactive compounds, with notable antioxidant and antifungal activities of *L. album* against *F. culmorum* and *F. proliferatum* both *in vivo* and *in vitro*, as evidenced by the inhibitory effects on mycelial growth, further confirmed by the reduction of ergosterol content and suppression of mycotoxin biosynthesis. Slight phytotoxic effects of *L. album* flower extracts on wheat seed germination and seedling growth were observed, highlighting the importance of considering potential adverse effects that may happen alongside the antifungal properties. As a result, this study paves the way for further exploration of natural plant extracts to promote sustainable wheat production and valorize locally available underutilized plants, contributing to the ongoing worldwide discussion on environmentally friendly disease management approaches.

The main conclusions confirming the research hypotheses can be presented as follows:

1. SC-CO_2 extraction with methanol as a co-solvent successfully extracted bioactive components from *L. album* flowers, yielding antioxidant-rich extracts. The extraction parameters, particularly temperature (50°C) and pressure (250 bar), significantly influenced the composition and antioxidant activity of the extracts. A strong correlation was observed between phenolic content and antioxidant capacity, emphasizing the significance of phenolic compounds in enhancing the antioxidative capabilities of the *L. album* extracts (H1).

2. In the *in vitro* study, *L. album* flower extracts exhibited strong antifungal activity against *F. culmorum* and *F. proliferatum*, with higher extract concentrations (10%) significantly inhibited fungal growth and mycotoxin biosynthesis, as indicated by reduced ergosterol and mycotoxin levels. While *F. culmorum* showed a clear concentration-dependent response, *F. proliferatum* did not follow the same trend, indicating species-specific differences in susceptibility to the extracts. These findings confirm the concentration-dependent antifungal efficacy of *L. album* flower extracts and susceptibility differences between species (H2).

3. While *L. album* extracts demonstrated antifungal activity, *In vivo* investigations revealed a minor phytotoxic effect of *L. album* flower extracts on wheat seed germination and seedling growth, particularly at higher concentrations. This finding highlights the need for a balanced approach when considering extracts for biological control, to avoid unintended negative effects on crop growth (H3).
4. The *in vivo* experiments confirmed the antifungal potential of *L. album* flower extracts in fighting against *F. culmorum* infections in wheat seedlings under controlled growth conditions and field trials. The extracts reduced fungal infections and mycotoxin accumulation in wheat tissues and harvested wheat grains, indicating their potential as a natural source of fungicides in agricultural practices (H4).
5. Wheat cultivars - Arkadia and Julius exhibited different responses to *Fusarium* infection and *L. album* extract treatment, with Arkadia confirming its greater resistance to *Fusarium* infections, this highlights the importance of genetic factors in plant-fungal interactions and underscores its critical role in developing effective disease management approach for crops.

These findings support the potential of *L. album* flower extracts as a natural antifungal agent in agricultural systems, although further research is needed to optimize their application and minimize phytotoxic effects.

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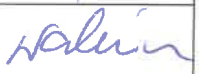
Statement about the contribution of authors

Publication P1

Uwineza, P.A.; Waśkiewicz, A. 2020. Recent Advances in Supercritical Fluid Extraction of Natural Bioactive Compounds from Natural Plant Materials. Molecules, 25, 3847. https://doi.org/10.3390/molecules25173847

Author	Author contribution	Percentage contribution	Signature
Pascaline Aimee Uwineza	Writing - original draft, Writing - review and editing	80%	
Agnieszka Waśkiewicz	Writing - review and editing	20%	

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Review

Recent Advances in Supercritical Fluid Extraction of Natural Bioactive Compounds from Natural Plant Materials

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Academic Editor: Teofil Jesionowski

Received: 24 July 2020; Accepted: 20 August 2020; Published: 24 August 2020

Abstract: In this review, recent advances in greener technology for extracting natural bioactive components from plant origin sources are discussed. Bioactive compounds of plant origin have been defined as natural chemical compounds present in small amounts in plants. Researchers have shown interest in extracting bioactive compounds because of their human health benefits and characteristics of being eco-friendly and generally recognized as safe. Various new extraction methods and conventional extraction methods have been developed, however, until now, no unique approach has been presented as a benchmark for extracting natural bioactive compounds from plants. The selectivity and productivity of traditional and modern extraction techniques generally depend on selecting the critical input parameters, knowing the nature of plant-based samples, the structure of bioactive compounds, and good scientific skills. This work aims to discuss the recent advances in supercritical fluid extraction techniques, especially supercritical carbon dioxide, along with the fundamental principles for extracting bioactive compounds from natural plant materials such as herbs, spices, aromatic and medicinal plants.

Keywords: bioactive compounds; supercritical extraction; supercritical fluids; co-solvent; essential oils; medicinal plants

1. Introduction

Extraction is one of the approaches used to isolate components from plant-based materials. At present, various extraction methods are used at the laboratory, pilot, and commercial scales by numerous researchers to extract the different target compounds present in plants [1]. Bioactive compounds are natural secondary metabolites extracted from various plant parts, such as leaves, stem, roots, seeds, flowers, and fruits, by using several extraction procedures [2,3]. Demand for these compounds has increased because they are perceived as natural and safe for applications in numerous industries such as cosmetics, food, feed, agriculture, and pharmaceuticals [3,4]. Bioactive compounds have been found to possess a wide spectrum of health-promoting properties for humans and animals such as antibacterial, antimicrobial, anti-inflammation, anti-aging, and anti-cancer effects [2,4–6].

Bioactive compounds—essential oils, carotenoids, fatty acids, phenolic acids, flavonoids—were conventionally extracted by steam distillation, solvent extraction, Soxhlet extraction, pressing method, and hydro-distillation, but they exhibit some limitations such as being too time-consuming, using too much organic solvents, losing some volatile compounds, degrading thermolabile compounds, the possibility of leaving toxic solvent residues in the extract, low yield, and low extraction efficiency [3]. Therefore, in recent years, green chemistry methods were developed for extraction purposes to reduce

energy and solvent consumption, reduce processing times, and replace conventional solvents with eco-friendly substitutes.

Modern approaches to extract and isolate bioactive compounds from plants-based materials are gaining attention in the research and development fields. For example, ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), and microwave-assisted extraction (MAE) [7–10] are currently accessible and environmentally sustainable technologies. According to Chemat et al. [11], greener technology is defined as "extraction methods based on the detection and development of extraction processes which will reduce energy consumption, enables the use of solvents substitutes, renewable natural products, and ensure a safe and high-quality extract/product" [11].

Ultrasound-assisted extraction is a mechanical technique based on sound waves, frequencies, and amplitudes that stimulate cell wall disruption and the discharge of cell content [8,10,12]. This process reduces the extraction time, is suitable for thermolabile and unstable compounds, improves the extraction yield, and reduces the solvent and energy consumption because of its effect on the transport mass, temperature, and solvent/sample ratio [8,10]. Pressurized liquid extraction is a solid–liquid technique consisting of the application of high pressure and temperature that raises the solvent's boiling point and induces its quick penetration into the sample matrix [7]. PLE contributes to a lower use of solvent, shorter extraction time, and increased extraction yield. However, it is not recommended for heat-sensitive compounds due to the high extraction temperature [7,10]. Supercritical fluid extraction is an advanced technique for extracting bioactive compounds by employing supercritical fluids as solvent. It has gained much attention over traditional methods due to its significant benefits, such as higher selectivity, diffusivity, and ecology [13].

Among the novel technologies mentioned, we discuss in this review supercritical fluid extraction (SFE) which is one of the best techniques for removing natural chemical components such as flavonoids, essential oils, seed oils, carotenoids, and fatty acids from natural plant materials, and it represents a sustainable alternative to traditional extraction systems [10,13]. Researchers have shown the considerable benefits of SFE over conventional techniques [3,9,10]. Carbon dioxide (CO_2) is an excellent solvent that has received particular attention in SFE because it is chemically inactive, economical, easily accessible, separable from extracts, non-toxic, and is an approved food-grade solvent [7,9]. Supercritical carbon dioxide (SC-CO_2) is a nonpolar solvent that is frequently used in SFE due to its gas-like and liquid-like properties, low critical temperature and pressure, and it has the selectivity and potentiality to extract heat-sensitive compounds. Furthermore, low polarity compounds and small molecules are easily dissolved in SC-CO_2, but large molecules and polar compounds are extracted with the addition of a co-solvent to enhance the extraction yield, which can be ethanol, methanol, or water. A particular focus should be placed on temperature and pressure during SFE because any alteration affects the entire process [3,13].

The objective of this paper was to provide an upgraded review on the claims of SFE by discussing the characteristics and principles of SC-CO_2, its applications in extracting natural bioactive compounds from plant-based materials, and its current use in different industries.

2. Characteristics of Supercritical Fluids as a Novel Extraction Technique

2.1. Background of Supercritical Fluid Extraction

SFE is classified among the novel extraction techniques that are a more environmentally friendly method by which to produce indigenous substances that have applications in various industries from sustainable sources such as herbs, spices, aromatic and medicinal plants. This advanced technology consists of the isolation/removal of targeted bioactive compounds through supercritical fluids.

In 1822, the initial discovery of the supercritical phase was realized by Baron Charles Cagniard de la Tour, who noticed changes in solvent behavior at a particular value of pressure and temperature [14,15]. The term "critical point" was coined by Thomas Andrews in 1869 as a result of his experiments on

the effects of temperature and pressure on as sealed glass tube of partly liquefied carbonic acid. He described it as the endpoint of the phase equilibrium curve which the critical temperature (Tc) and critical pressure (Pc) reached when the existence of two phases disappeared [10,14]. A few years later, Hannay and Hogarth discovered the SFE method application and the fundamentals of this technology using CO_2 in the supercritical state were developed in 1960 [16]. The earliest practical application of supercritical fluids was the decaffeination of green coffee beans started in Germany; after a few years, the extraction of oils from hops using liquid CO_2 was developed in Australia [17]. By the 1980s, industrial applications of both technologies were developed and effectively adopted in different countries [16]. Currently, various products are being produced using the technology and are accepted all over the world.

2.2. Concept of Supercritical Fluids Extraction and Principles

Basically, a simple SFE process comprises extraction and separation as the essential steps [17]. During extraction, either solid or liquid samples may be used, depending to the system settings, but solid samples are more used compared to liquid samples. Regarding solid samples, columns are filled with pre-treated (dried and milled) samples, and the pressurized supercritical solvents flow through the column and dissolve extractable compounds from the solid matrix. The dissolved compounds are transported by diffusion out to the separator where the mixtures of extract and solvent are separated through pressure reduction, temperature increase, or both [17–19].

Many companies are now commercially producing SFE equipment at varying scales according to their intended use, such as for use in the laboratory, pilot studies or industrial use. The most simple system consists mainly of a chiller used to cool the solvent gas, a solvent pump that pushes the fluid throughout the system, an extraction column that holds the samples to be extracted, separators which collect the extract, heat exchangers for adjusting the temperature of process materials, an oven utilized to keep the extraction column above the critical temperature of the extraction fluid, and a back pressure regulator used to maintain the pressure in the system above the critical pressure of the fluid [20,21]. SFE can be implemented in two different modes—dynamic and static—which can be used separately or combined during extraction. In dynamic mode, the supercritical fluid flows steadily through the extraction column containing the sample, while in static mode, the sample absorbs the supercritical fluid and there is no run-off fluid from the extraction column during the process [22].

Most of the time, co-extraction of unwanted compounds occurs during the extraction process, which might result in a poor-quality extract. Thanks to advanced technology, this problem can be solved by conducting SFE with a fractional separation process, allowing the improvement of selectivity in SFE [23]. This concept can be conducted in multiple steps during separation, by coupling some separators in series and adjusting processing conditions, such as pressure and temperature, according to the equilibrium solubilities of the targeted compounds [24].

During SFE, different variables, such as extraction temperature, pressure, type, percentage of co-solvent, and the sample size must be optimized to enhance the extraction yield of targeted compounds. Additionally, the solubility and mass transfer resistance of raw materials are associated with those variables [25–27]. The solubility of an extractable compound must be maximized in SFE because it is one of the main factors influencing the effectiveness of extraction and the quality of extract [13]. It has been reported that the solubility of the solute in SFE depends on temperature and pressure, which have an effect on the density of fluid. SFE is a convenient technique because it enables an adjustment of the solvent power or selectivity of the supercritical fluids (SCF), and it can be directly connected with gas chromatography (GC) or supercritical fluid chromatography for analytical purposes [27]. Various studies have shown numerous benefits of SFE including its fast processing time, suitability for extracting volatile and thermolabile compounds, higher productivity in terms of increased yields, reduced solvent use, and protection of the environment by using safe solvents. It plays a vital role in different areas such as food, pharmaceutical, agriculture and cosmetics [18]. As discussed, nowadays SFE is not only applied in laboratories for research purposes but its application has been

developed commercially at an industrial scale, for example, to produce natural food ingredients (hops, aromas, spices, colorants, vitamin-rich extracts, specific lipids), nutraceuticals, pharmaceuticals, and to remove pesticides from food products [27,28]. The use of supercritical fluid is characterized by different properties, namely, density, viscosity, and diffusivity, which can be changed to improve its transport properties.

2.3. Properties of Supercritical Fluids

Supercritical fluids are chemical solvents that can be compressed above their critical point, are generally considered environmentally friendly, and are commonly used in the extraction process because they provide excellent results due to their unique characteristics [29]. SCF is used as a replacement for organic solvent in laboratory processes and various industries such as food, pharmaceuticals, agriculture, and cosmetics. As shown in Table 1, many compounds have been considered as SCFs, for example, hydrocarbons (pentane, butane, hexane), aromatics (benzene, toluene), alcohols (methanol, isopropanol, *n*-butyl alcohol) and some gases (carbon dioxide, ethylene, propane) [18]. Among the abovementioned SCF, CO_2 is unquestionably the most often employed solvent due to its numerous different benefits [25,29].

Table 1. Chemical solvents employed in supercritical fluids extraction (SFE) and their critical characteristics [30].

Solvent	Molecular Weight	Critical Temperature	Critical Pressure	Critical Density
	[g/mol]	[K]	[MPa]	[g/cm^3]
Carbon dioxide	44.01	304.1	7.38	0.469
Water	18.02	647.3	22.12	0.348
Methane	16.04	190.4	4.60	0.162
Ethane	30.07	305.3	4.87	0.203
Propane	44.09	369.8	4.25	0.217
Ethylene	28.05	282.4	5.04	0.215
Propylene	42.08	364.9	4.60	0.232
Methanol	32.04	512.6	8.09	0.272
Ethanol	46.07	513.9	6.14	0.276
Acetone	58.08	508.1	4.70	0.278

A fluid is regarded as supercritical when its pressure and temperature are beyond its critical points, that is, critical pressure (Tc) and critical temperature (Pc). In this state, the fluid is represented by both its gas and liquid phase properties in an advantageous way. Their actions are close to that of gas in some ways, but close to liquid in others [30].

For example, a simple CO_2 phase diagram can demonstrate the concept of the supercritical state (Figure 1). CO_2 usually exists in three physical states (solid, liquid, and gas) due to variations of pressure and temperature [29], but sometimes there is a fourth CO_2 phase—supercritical fluid—that is the result of the dynamic equilibrium that appears at the point where there are no differences between liquid and gas phases [31,32]. The phase diagram below shows different points and states of CO_2 in terms of combinations of high or low temperature and pressure. The triple point on this diagram is the point that represents all three phases of CO_2 together in equilibrium (−56.6 °C and 51.1 bar) [32]. The critical point appeared at 31.1 °C and 78.3 bar, which are the Tc and Pc of CO_2, respectively [25,29,30]. The region above the critical point is a supercritical fluid region which appears when there is no physical difference between a gas and a liquid, and over that critical point no matter how much pressure or temperature is increased, the substance cannot be changed from liquid to gas or from gas to liquid [30,32].

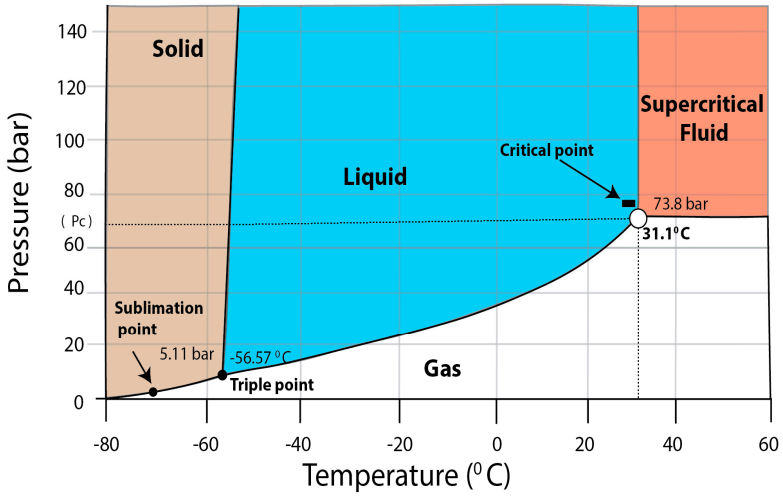

Figure 1. Scheme p-T representation of the CO_2 phase based on Gopaliya et al. [32] with own modifications.

Briefly, the supercritical region can be attained through two approaches: (i) by increasing the pressure above the Pc-value of the substance, while maintaining a stable temperature and then extending the temperature above the Tc-value at a stable pressure value; or (ii) by increasing the temperature above the Tc-value first and then increasing the pressure above the Pc-value.

Supercritical fluids generally display physicochemical properties between those of a liquid and gas, typically characterized by low viscosity, high density, and diffusivity, which can be managed by changing the pressure and/or temperature (Table 2) [29,33,34]. Those properties are predominant during the SFE process design and make them an excellent solvent for different applications [17] due to their penetrative power, which is based on the high mass transfer rate and high density of fluids in the extractable components.

Table 2. Comparison of physicochemical properties of gases, supercritical fluids, and liquids [30].

State	Density [kg/m^3]	Viscosity [μPa]	Diffusivity [mm^2/s]
Gases P = 1 atm, T= 21 °C	1	10	1–10
Supercritical fluids P = Pc, T = Tc	100–1000	50–100	0.01–0.1
Liquids P = 1 atm, T = 15–30 °C	1000	500–1000	0.001

P = pressure, T = temperature.

The density of a supercritical fluid is between that of a gas and a liquid but is closer to that of a liquid. It mostly relies on pressure and temperature conditions: When pressure rises at a constant temperature, it increases the density of a supercritical fluid and solvating power. On the other hand, when the temperature rises at a constant pressure, it reduces the density of a supercritical fluid and solvent strength [32]. Therefore, this is the main characteristic and fundamental source of the excellent dissolving properties of fluid with a solute molecule, which is very strong.

The viscosity of a supercritical fluid is one of the thermophysical properties of SCF which is generally known to be low and nearly equal to that of a gas but less than that of a liquid [17]. This low viscosity enhances the penetration power of SCF due to the lower resistance than that of a liquid to a solute. Temperature has a higher impact on SCF viscosity than liquids [32].

The diffusivity of a supercritical fluid is typically higher than in a liquid and lower than in a gas [17]; consequently, the solute can show better diffusivity in a SCF than in a liquid. Moreover, temperature and pressure conditions show different effects: as the pressure increases, the diffusivity in a SCF decreases and when the temperature increases, the diffusivity also increases. This property enhances the capacity of SCF to be a suitable solvent for analytical purposes [32,33].

As mentioned previously, these properties are connected, and make SFE stand out as an excellent technique. For example, low viscosity and high diffusivity allow better transport properties than liquids; they diffuse effortlessly in a solid sample and increase efficiency and extraction yield. Additionally, high density combined with solvent power can be tailored automatically, and this can give high solubility and high selectivity to the SCF; thus, new compounds can be extracted and employed in the development of new products [17].

3. Operational Use of Carbon Dioxide in Bioactive Compound Extraction

Carbon dioxide is extensively used as the supercritical fluid in various fields for extraction purposes due to its critical properties (Tc = 31.1 °C; Pc = 73.8 bar), which makes it an excellent solvent for extracting bioactive compounds sensitive to heat [29]. The unique solvent properties of supercritical carbon dioxide have made it a desirable compound for separating antioxidants, pigments, flavors, fragrances, fatty acids, and essential oils from plant and animal materials. SC-CO_2 has numerous benefits. It is a cheap and readily available in large quantities with a high degree of purity [28], furthermore it is an environmentally friendly substitute for organic solvents and is designated as a safe solvent by different organizations such as the U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) [35,36].

A further advantage of SC-CO_2 is its ability to produce extracts free of solvent residue because CO_2 is a gas at ambient temperature and pressure, so it can use a low amount of solvent and minimize thermal damage to bioactive compounds due to its low critical temperature.

CO_2 has exciting features as a solvent because it has high diffusivity, it is safe for human health and the environment, reusable, inert, non-toxic, non-flammable, and non-corrosive [25]. CO_2 is useful in supercritical fluid extraction processes because being close to the ambient environmental conditions it can readily penetrate through plant material and dissolve the targeted extracts. However, its extractability with substances depends on the occurrence of specific groups in the structure, as well as their polarity and molecular weight [13,35,37]. Additionally, the extract composition for volatiles or non-volatiles can be changed easily by modifying the pressure and temperature conditions.

CO_2 is gaseous at ambient pressure and temperature, which makes analyte recovery quite simple and provides solvent-free analytes. However, it has a limited solubility towards compounds because it is regarded as non-polar [38]. Studies have shown that hydrocarbons and other organic compounds with relatively low polarity and molecular weights (MWs) under 250 exhibit excellent solubility in SC-CO_2. For example, esters, aldehydes, ethers, ketones, lactones, and epoxides are easily extractable in SC-CO_2, so the process can be carried out at lower pressures (75–100 bar). Moderately polar components with an MW in the range of 250–400, such as benzene derivatives, substituted terpenes, sesquiterpenes, or oleic acid, are moderately soluble in SC-CO_2, and a higher pressure is required for their extraction. Highly polar substances (MW over 400) that contain carboxylic acids and three or more hydroxyl groups (e.g., sugars, tannins, proteins, waxes, carotenoids, or pesticides) are almost insoluble in SC-CO_2 [13,17]. To obtain the targeted compounds, a polar co-solvent such as methanol, ethanol, and water is generally used to enhance the dissolvability of polar compounds in the supercritical mixture and improve the selectivity of SC-CO_2 [25,38].

4. Parameters to Consider and Their Effect during Supercritical Fluid Extraction

During SFE, the choice of working conditions depends on the target compounds to be extracted; however, the parameters of temperature, pressure, co-solvent concentration, extraction time, and particle size are the most prominent and should be taken into consideration for efficient extraction.

4.1. Effects of Temperature and Pressure

The extraction requirements of temperature and pressure are the primary parameters that influence the extraction efficiency because of their effect on the solubility of a substance during SFE. Generally, an increase of pressure at a specific temperature in the process increases the density of the solvent

and the solubility of targeted compounds [28,39]. Therefore, the higher the pressure, the smaller the solvent volume needed for a particular extraction [28]. Nevertheless, elevating pressure to a given point can reduce the extract's antioxidant activity and decrease the diffusivity of the SCF, which reduces solute dissolution [40]. Furthermore, a high pressure is not suggested for all substances and targeted compounds because it can result in a compacted raw material, which can adversely affect the extraction yield [41]. The extraction temperature at constant pressure has two opposing effects during SFE. Increased temperatures decrease the solvent density, thus reducing its solvating power, but it improves the vapor pressure of desired compounds, consequently increasing the analyte solubility and extraction yield. However, because increased temperature decreases the solvent density, this reduces its solvating power and analyte solubility, negatively affecting yield [28,40,42]. These two opposite effects may result in the cross over of the isotherms, in a phenomenon known as retrogradation, where the solubility of solute does not depend on the density of solvent [43]. Therefore, according to the literature, the supercritical fluid extraction temperature of thermolabile compounds has to be fixed between 35 and 60 °C to avoid degradation [27], and the pressure should be around 400 bar. These conditions should be carefully selected based on the purpose of the process and the nature of the targeted compounds [15].

Studies on the influence of temperature and pressure on the extraction process of bioactive substances gave different results due to the plant materials and targeted compounds [40,42,44–48]. For example, high pressures were reported to enhance the global extraction yield of phenolic compounds [40,45,47], while Akay et al. [46] reported a high total phenolic content under low pressure and no significant difference to the yield when increasing temperature and pressure. Some results showed that an augmentation in the recovery of phenolic compounds could be obtained by reducing the extraction temperature and increasing the addition of co-solvent and pressure [45].

4.2. Effects of Co-Solvent/Modifier

By definition, a co-solvent is an organic solvent that, when added at various proportion to CO_2, may dissolve with the supercritical fluid, and can retain a considerable solvent power towards the targeted compounds [49]. As previously reported, CO_2 is the most frequently used supercritical fluid in various industries, but its low polarity restricts its use for the extraction of lipophilic and polar compounds [15]. To overcome this problem, polar co-solvents such as ethanol, methanol, water, acetic acid, formic acid, and many other polar or nonpolar co-solvents, can be used during the extraction to improve the solvation power of SC-CO_2, enhance its affinity for poorly soluble solutes (alkaloids, phenolics, and glycosidic compounds), increase solubility, and increase the extraction yield [25]. Among the listed co-solvents, methanol and ethanol are frequently used at concentrations below 10% of the quantity of CO_2 employed for the extraction [15,37]. However, ethanol is less toxic than methanol. The best co-solvent for a specific extraction process should be determined through preliminary experiments, and the type of sample, targeted compounds, and its final application should also be considered.

Different researchers have shown that the co-solvent can affect not only the total extraction yield but also the bioactive properties of the extracts such as total phenolics extraction yield, antioxidant and anti-inflammatory activities of extracts [50–52]. The percentage and type of co-solvent are significant in the extraction. Both are crucial factors affecting the solubility of targeted compounds in SFE. However, the addition of a large volume of co-solvents can modify the critical parameters of the solution and can reduce the selectivity [51]. In SFE, there are three different ways that co-solvents can be used in the system: as a mixed fluid in the pumping system; by injecting the co-solvent as a liquid into samples before extraction; and as a cylinder tank of pre-modified CO_2, although this method is expensive and seldom used [41].

Several scientists have studied the effect of co-solvent on SC-CO_2 extraction, and their results have various implications. For instance, in terms of the carotenoid composition of pumpkin in SC-CO_2 extraction with and without co-solvent, co-solvent increased the total carotenoids yield from pumpkin.

Shi et al. [47] demonstrated that by increasing the polarity of CO_2 with ethanol, it is possible to increase the total carotenoid yield by 1.8 times. In other studies, Uquiche et al. [51] investigated the impacts of ethanol concentration and pressure on the phenolics extraction yield, total extract yield, antioxidant and anti-inflammatory activities by using supercritical extraction of *L. rivularis* stalks. The result showed that the highest total extract yield, phenolics extraction yield, and antioxidant activity were achieved with low use of co-solvent, while anti-inflammatory activity improved with a greater addition of ethanol. Klein et al. [53] evaluated the effects of the addition of two different co-solvents (ethyl acetate and ethanol) and the operational pressure on the SC-CO_2 extraction process, and the results revealed that the addition of ethanol as a co-solvent resulted in higher yields than those obtained using ethyl acetate.

4.3. Effect of the Raw Matrix on Supercritical Fluid Extraction

Several elements, such as the nature of the raw material, moisture content, particle size, shape, surface area, and porosity are crucial elements that influence solubility and the mass transfer process during SC-CO_2 extraction [13]. The correct selection of these elements can produce the complete extraction of the targeted compounds in a shorter time [27].

Typically, the sample material to be extracted must be dried to reduce the moisture content, as the water content in the sample can compete with the extractable solute to associate with the solvent and reduce the extraction yield; nevertheless, in some cases the presence of water is needed to permit the good interaction of solvent with the solute. The recommended moisture content should be between 4–14% [18]. The particle size of solid samples and porosity have a direct impact on the mass transfer rate in the process. Reducing the particle size increases extraction efficiency because diminishing particle size reduces the diffusion path of the solvent and increases the contact surface area which resulted in the acceleration of extraction process [15,18,41]. However, it is necessary to avoid overly small particles because they can increase the internal mass transfer resistance, which provokes channeling within the column and a reduction of efficiency and yield during the extraction process [15,18,27]. According to the literature, the mean particle size for SFE of natural products varies from 0.25 to 2.0 mm [15,18]. Different researchers studied the effect of particle size during SC-CO_2, and Del Valle and Uquiche [54] reported that the oil yield was dependent on the particle size of the rosehip seeds. Aris et al. [55] investigated the effect of particle size on *Momordica charantia* extract yield with different mean particle sizes of 0.2, 0.3, 0.5 and 0.7 mm under constant operating conditions (200 bar, 65 °C and 4 mL/min), and showed that a reduction in the particle size could increase the yield. They concluded by showing that 0.3 mm was the best mean particle size to produce the highest *Momordica charantia* extract yield. Kehili et al. [56] investigated the influence of particle size of tomato peel on the oleoresin extraction yield, and ground tomato peels of 0.3 mm particle size and unground tomato peels of 1 mm particle size were used for SFE at 50 °C, 400 bar and a CO_2 flow rate of 4 g/min for 105 min using a batch of 10 g. Their findings showed that the particle size of tomato peels affects the kinetics and yield of oleoresin extraction, and they concluded that the extract was enhanced by the smaller particle size of the dried tomato peels.

4.4. Influence of Extraction Time

Extraction time is important for SFE because it can affect the extract's composition. When the extraction time is short, it can lead to incomplete extraction. However, if the extraction time is too long, it would result in time and solvent wastage, as well as bioactive compound degradation [41]. The extraction time depends on flow rate: When the flow rate is high, the extraction time is short. A preliminary study should be performed to determine the optimal time and fluid flow rate to obtain the highest extract yield [50].

5. SFE in Plant Bioactive Compounds Extraction

The use of natural substances in various industries such as food, pharmaceutical, and cosmetics has gained considerable attention recently due to their confirmed effects by the scientific researcher as sustainable alternatives to synthetic compounds, their perceived safety to the consumer, and because they are environmentally friendly.

Plants symbolize a significant resource of chemical components that are historically used as natural remedies for some diseases and now those components are used in food additives production (e.g., natural flavor, aroma, and color), the creation of functional foods, production of natural pesticides, and development of new drugs [3]. Plant bioactive compounds are natural non-nutritive components of plant food biosynthesized as secondary metabolites [5]. They can provide essential benefits or undesirable effects on humans or animals and are found in small concentrations but are economically significant [57]. Plant bio-actives are usually identified in plant foods such as fruits, vegetables, grains, and non-food plants such as herbs, spices, aromatics, and, sometimes, in plant waste materials from factories. Even though they are not recognized as essential components, different experiments showed that they have a role in enhancing human health.

Different bioactive compounds extracted from natural sources by using supercritical CO_2 extraction have been shown (Table 3) to exhibit antimicrobial, antioxidant, antiseptic [58,59], antibacterial, antifungal [60], antiviral, anti-inflammatory [2], antitumor [61], anti-obesity, anticholinesterase, phagocytotic, and therapeutic values [62,63]. They can also play the role of functional food constituents such as coloring, flavoring, preserving food additives, fragrances, authenticity indices, and biomarkers in metabolomic pathways. The synergism in some of the bioactive compounds enhances their bioactivity [64].

As the extraction process is the leading method to recover bioactive compounds, supercritical CO_2 extraction has been reported as the best modern technology to sustainably and safely extract bioactive compounds.

Table 3. Selected bioactive compounds and their bioactivities.

Compounds	Plant Materials	Bioactivities	References
Geranylgeraniol	Annatto seed (*Bixa Orellana*)	anti-inflammatory activity, regulation of testosterone production, action against Chagas disease and leishmaniasis, and anti-cancer activity	[65]
Curcumin	Turmeric rhizomes (*Curcuma longa* L.)	antioxidant, antimalaria, antimicrobial, anti-viral properties, fungicidal activity, anti-Alzheimer anti-mutagenic, and anti-carcinogenic qualities	[66]
Thymol	Thyme (*Thymus praecox Polytrichus*)	antibacterial, antifungal, anti-inflammatory, antioxidant activities, local anesthetic	[67]
Eugenol	Purple basil (*Ocimum basilicum*)	antioxidant, antibacterial and antimicrobial activities	[68]
Carvacrol	*Satureja montana* L.	antioxidant, antiproliferative, and anti-cancer	[69]
Linalool	*Coriandrum sativum* L. and *Ocimum basilicum* L.	antimicrobial, anti-carcinogenic, antioxidant and antidiabetic activities	[69]
Camphor	Sage (Sage *officinalis*)	anti-inflammatory and anti-atherogenic	[70]
Xanthohumol	Hops (*Humulus lupulus*)	antibacterial activity anti-cancer	[71]
Artemisinin	*Artemisia annua* L.	antimalaria, antiulcerogenic, antifibrotic and antitumoral activity	[72]

According to biochemical pathways and chemical classes, plant bioactive compounds are biosynthesized from four primary pathways: the shikimic acid pathway, malonic acid pathway, mevalonic acid pathway, and methylerythritol pathway [3,73]. From the pathways mentioned

above, plants' natural bioactive compounds can be categorized into three main classes: alkaloids, terpenoids/terpenes, and phenolics [5,73,74]. A simplified figure of diverse pathways for the biosynthesis of the three major groups of plant bioactive compounds is shown in Figure 2.

Plant bio-actives are found in an enormous range of foods consumed as part of the human diet such as fruits, vegetables, chocolate, wine, tea, and coffee. However, those used in various industries can be extracted from different parts of plants such as leaves, stems, roots, tubers, seeds, buds, fruits, and flowers [2].

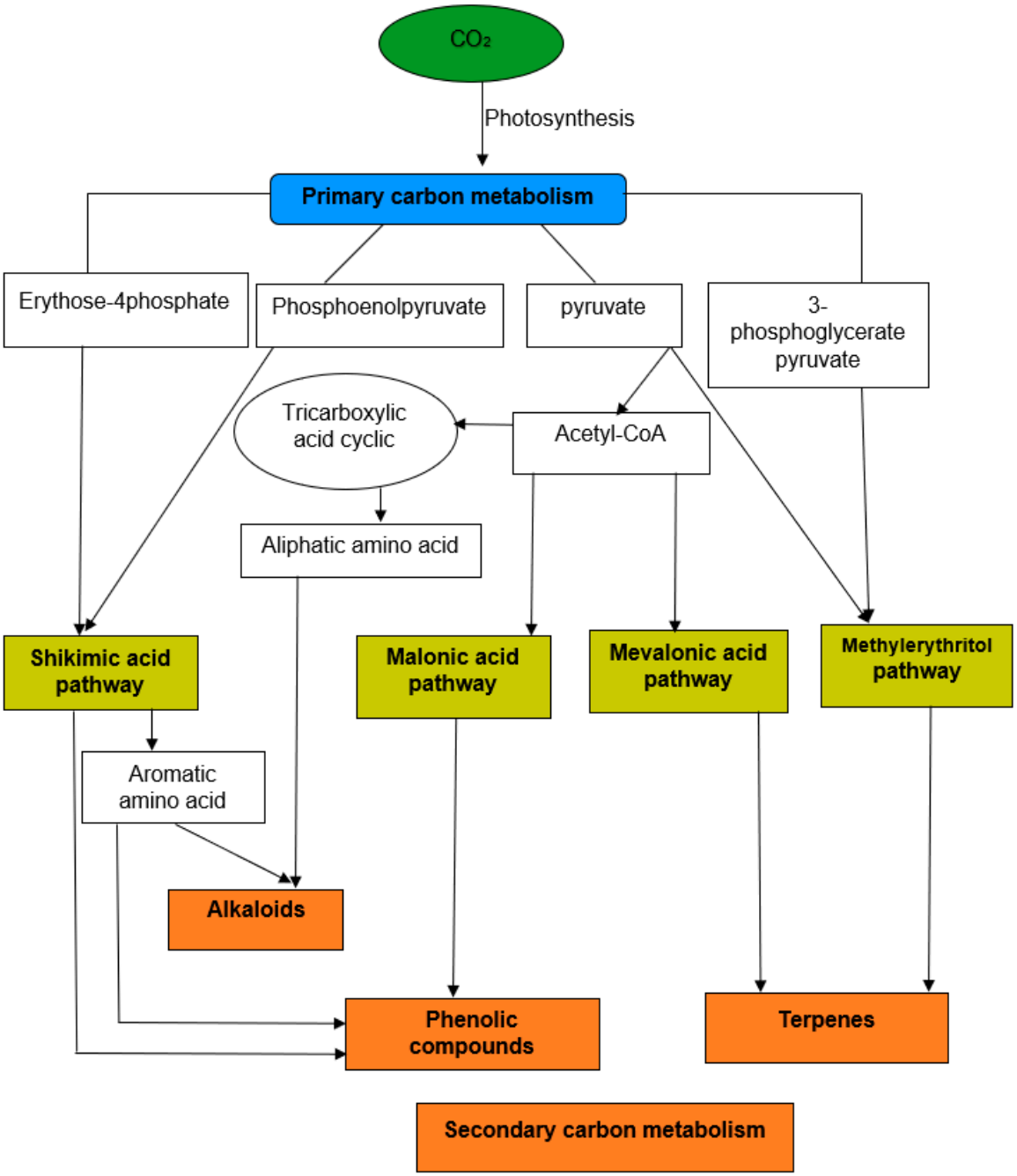

Figure 2. A general overview of the biosynthetic pathways involved in the biosynthesis of secondary metabolites (main categories of bioactive compounds) and their inter-relationships with primary metabolism in plants [3].

5.1. Alkaloids

Alkaloids are secondary plant metabolites, which are also found in microbes and animals. Structurally, they consist of carbon, hydrogen, nitrogen, and occasionally oxygen. Their alkaline nature is due to their nitrogen content [58]. There are over 12,000 known alkaloid compounds with low molecular weight, which have effects in the defense of plants against herbivores and pathogens. Some alkaloids exhibit significant bioactivities that has led to their exploitation as pharmaceuticals, stimulants, narcotics, and poisons [58,73]. Each plant species usually comprises a few alkaloids, but the most well-known alkaloids are morphine, codeine, caffeine, nicotine, cocaine, and vinblastine [73].

Caffeine is an excellent example of an alkaloid, commonly known to be present in different soft beverages, drinks, and some natural plants such as coffee beans, cocoa beans, mate leaves, guarana seeds, kola nuts, and tea leaves [75]. Several years ago, caffeine was employed as one of the ingredients in the production of some pharmaceuticals. However, various studies have indicated the side effects of caffeine consumption in humans. For example, insomnia, nervousness, irritability, anxiety, hostility, and mood swings, and sometimes it causes health problems in pregnant, children, and some patients [75]. These adverse consequences have resulted in the increased consumption of decaffeinated products that causes the evolution of various techniques that eliminate this alkaloid from coffee beans. Among the various techniques investigated, supercritical CO_2 has been reported as an effective modern process to extract and eliminate caffeine from coffee, tea, and other products [63]. According to different research (Table 4), the extraction of alkaloids by using SC-CO_2 extraction has been considered a quick and effective process compared to the conventional technique.

Table 4. Application of supercritical CO_2 for the separation of alkaloids from plants.

Compounds	Plant Material	SFE Parameters	Country	References
Pyrrolidine	Leaves of *piper amalago* (Piperaceae)	Pressure: 150, 200, and 250 bar Temperature: 40, 50, and 60 °C Co-solvents: ethanol, methanol and propyleneglycol 5% (*v*/*v*) Extraction time: 20, 40, and 60 min CO_2 flow rate: 3 mL/min Particle size: 0.757 mm	Brazil	[76]
Olchicine, 3-demethylcolchicine, colchicoside	Seeds of wild plants of *Colchicum autumnale* L. (Colchicaceae)	Temperature: 25, 30, 35 and 40 °C Co-solvent: methanol 3% Extraction time: 0 to 25 min static extraction and 0 to 30 min dynamic extraction Density: 0.80, 0.85 and 0.90 g/mL CO_2 flow rate: 1.5 mL/min	Spain	[59]
Total alkaloids, peimine, peiminine	Flower of Fritillaria thunbergii Miq	Pressure: 150–350 bar Temperature: 50–70 °C Co-solvent: ethanol and water ratio 80:100 (*v*/*v*) Extraction time: 90–210 min	China	[62]
Caffeine	Guayusa leaves (*Ilex guayusa* Loes)	Pressure: 150, 200 and 250 bar Temperature: 45, 60 and 75 °C Extraction time: 180 min CO_2 mass flow: 8.3 g/min	Brazil	[60]
	Tea stalk and fiber waste	Pressure: 150–300 bar Temperature: 50–70 °C Co-solvent: ethanol 1–7 % Extraction time: 60–300 min CO_2 flow rate: 10 g/min Particle size: 0.2–0.6 mm	Turkey	[77]
	Green coffee beans	Pressure: 152, 248 and 352 bar Temperature: 50 and 60 °C Co-solvent: ethanol and isopropyl alcohol 5% (*v*/*v*) CO_2 flow rate: 1.8 g/min	Brazil	[78]
Vinblastine and vincristine	*Catharanthus roseus*	Pressure: 300 bar Temperature: 40, 50, and 60 °C Co-solvent: ethanol 2, 5 and 10% (*v*/*v*)	Brazil	[79]

From the analysis of studies on alkaloid extraction with SC-CO_2, the conditions of the parameters to be taken into consideration were different due to the plant material and working conditions.

A pressure of 300 bar, temperature of 60 °C, and using ethanol as the co-solvent were considered the optimal parameters for that extraction.

5.2. Terpenes or Terpenoids

Terpenoids are among the most prominent groups of secondary metabolites, and over 25,000 members of this group have been reported [74]. The chemical structure of terpenoids is formed by head to tail rearrangement of two or more isoprene molecules. Isoprene is a basic structure of the terpenoids, made of a branched five-carbon unit synthesized from acetyl-CoA or 3-phosphoglycerate [80].

Repetitive combination of the five-carbon unit of isoprene generates numerous types of terpenoid molecules such as hemiterpene (C_5), monoterpene (C_{10}), sesquiterpene (C_{15}), diterpene (C_{20}), triterpene (C_{30}), and tetraterpene (C_{40}), etc. [80,81]. Terpenoids play essential physiological roles in plants including growth, development, and defense. Different terpenoids have been extracted using SC-CO_2 (Table 5) and their applications are great and varied across industries.

Table 5. Examples of terpenoids extracted by supercritical CO_2.

Targeted Extract	Plant Materials	SFE Parameters	Country	References
Essential oils	*Piper auritum*	Pressure: 103.4 and 172.4 bar Temperature: 40 and 50 °C	Mexico	[82]
	Lippia graveolens, (Mexican oregano leaves)	Pressure: 130, 150 and 350 bar Temperature: 40 and 60 °C	Mexico	[48]
Essential oils	Spearmint leaves	Pressure: 85–120 bar Temperature: 38–50 °C, CO_2 flow rate: 0.059–0.354 g/min Particle size: 0.177–2 mm Dynamic extraction time: 20–120 min	Iran	[83]
	Roots of vetiver grass	Pressure: 145 bar Temperature: 45 °C Co-solvent: ethanol (0, 5, 10 and 15%)	Australia	[84]
	Clover leaf extract	Pressure: 150, 185 and 220 bar Temperature: 40, 50 and 60 °C	Brazil	[85]
	Rosemary (*Rosmarinus officinalis*)	Pressure: 103.4 and 172.4 bar Temperature: 40 and 50 °C Particle size: 0.6 mm	Mexico	[86]
	Ruta chalepensis	Pressure: 100, 150, 220 bar Temperature: 40 °C Extraction time: 30 min static extraction followed by 220 min of dynamic extraction	Tunisia	[87]
	Echinophora platyloba	Pressure: 80–240 bar Temperature: 35–55 °C Dynamic extraction time: 30–150 min Particle size: 0.30–0.90 mm	Iran	[88]
Raspberry seed oil	Raspberry seed	Pressure: 250, 300 and 350 bar Temperature: 40, 50 and 60 °C CO_2 flow rate: 3, 5 and 6 g/min, Particle size: 0.2–0.4 mm Extraction time: 240 min	Serbia	[89]
Apple seed oil	Apple seed	Pressure: 300, 500, 750, 1000 and 1300 bar Temperatures: 43, 53 and 63 °C	New Zealand	[90]
Carotenoids	Nantes carrots peels	Pressure: 150, 250 and 350 bar Temperature: 50, 60 and 70 °C Co-solvent: ethanol 5, 10 and 15% (*v*/*v*) CO_2 flow rate: 15 g/min Extraction time: 80 min	UK	[91]
	Flesh and peels of sweet potato, apricot, tomato, peach and pumpkin, and the flesh and wastes of green, yellow and red peppers	Pressure: 350 bar Temperature: 59 °C Co-solvent: ethanol 15.5% (*v*/*v*) CO_2 flow rate: 15 g/min Extraction time: 30 min	UK	[92]
	Mango peels	Temperature: 40–60 °C Pressure: 250–350 bar Co-solvent: ethanol 5–15% (*v*/*v*) CO_2 flow rate: 6.7 g/min Extraction time: 180 min	Colombia	[93]

Table 5. *Cont.*

Targeted Extract	Plant Materials	SFE Parameters	Country	References
Germacrene (sesquiterpene)	Leaves of *Piper klotzschianum*	Pressure: 180, 200, and 220 bar Temperature: 40, 60, 80 °C Co-solvent: methanol, ethanol, isopropanol (1, 3, 5%)	Brazil	[94]
Green coffee oil	Green coffee beans	Pressure: 200–400 bar Temperature: 40–60 °C Co-solvent: ethanol 0–5.7% (*v*/*v*)	Brazil	[95]
Vouacapan (diterpenes)	Sucupira fruits (*Ptedoron* spp.)	Pressure: 100–220 bar Temperature: 40–60 °C	Brazil	[96]
Oxygenated monoterpenes (camphor, 1,8-cineole), α-humulene, viridiflorol, and manool	*Salvia officinalis* L. (sage) leaves	Pressure: 100–300 bar Temperature: 40–60 °C Extraction time: 90 min	Croatia	[97]
Lycopene	Tomato peel by-product containing tomato seed	Temperature: 70–90 °C Pressure: 20–40 bar Particle size: 1.05 ± 0.10 mm CO_2 flow: 2–4 mL/min Extraction time: 180 min	Japan	[98]
Geranylgeraniol	Annatto seed	Pressure: 100, 170, 240 and 310 bar Temperature: 40 and 60 °C CO_2 densities: 290–915 kg/m^3	Brazil	[65]
Artemisinin	*Artemisia annua* L.	Pressure: 100 bar Temperature: 40 °C CO_2 flow rate: 13.3–20 g/min	Italy	[72]

In reference to studies on terpenoid extraction with SC-CO_2, the parameters to be taken into consideration can be different due to the plant material and working conditions, but the temperature should be around 40 °C and the pressure should be 250 bar to obtain the highest yield, although the use of different co-solvents was not considered in some studies.

Since ancient times, terpenoids have been widely used by humans. Some monoterpenes and sesquiterpenes are used as food additives, and flavor and fragrance agent are added to food products, beverages, perfumes, soaps, toothpaste, tobacco and other product [81]. For example, carotenoids are a well-known class of tetraterpene plant pigments; they are responsible for the yellow-red colors of various plant organs, and they are used in the food industry as natural food colorants to make the food product color more appealing to consumers. Carotenoids are among the most important bioactive compounds which can enhance human health due to their pro-vitamin A activity, anti-cancer activities, and antioxidant power [92,99]. Carotenoids from various vegetables matrices, such as pumpkins, tomatoes, carrots, apricot, peach, sweet potato, green, yellow, and red peppers, have been successfully extracted by supercritical carbon dioxide [92]. In recent years, the demand for carotenoids has increased extensively because of the growth in its application as animal feed, dietary supplements, pharmaceuticals, food and beverages, cosmetics, etc. [92]. Carotenoid demand is expected to record an annual increase of 4% from 2018 to 2023 and exceed a value of the USD 2 billion by 2023 worldwide. The primary market is in Europe, representing 42% of the total, followed by North America and Asia, which represent 25% and 20% of the market, respectively [99,100].

Essential oils are among the most important compounds to have been extracted by supercritical CO_2 from various species of herbs and aromatic plants, and they are generally composed of terpenes and terpenoids. They are used to produce food additives, cosmetics, herbicides, insecticides, medicines, fragrance in perfumery, and in aromatherapy [82]. Typically, essential oils can be found in every part of plants, however, their composition and effects may vary with the variety of the plants. Essential oils are always produced in small quantities, and their extraction yields can vary between 0.5–6%, but their positive effects do not require large quantities [101]. Essential oils have numerous beneficial properties including antifungal, anti-inflammatory, antibacterial, and antioxidant activity [102]. The antimicrobial effect of essential oils can be associated with their ability to penetrate the bacteria cell wall and inhibit its functional properties [84]. This ability is due to its hydrophobicity, which allows essential oils to separate the lipids from the cell membrane and cause an increase in cell permeability.

Currently, both academia and pharmaceutical industry are highly interested in essential oil extraction due to their pharmacological characteristics. The insecticidal activities of essential oils are also of interest to agricultural scientists and agri-businesses. Moderate pressures (90–120 bar) and temperatures (35–50 °C) are desirable to dissolve the essential oil compounds during extraction [102].

5.3. Phenolic Compounds

Phenolic compounds, also known as phenols, are secondary natural metabolites generally found in the plant kingdom (they are rarely present in bacteria, fungi, and algae) [103]. Approximately 8000 types of phenolics are known, for example, flavonoids, stilbenes, lignans, resveratrol, and gallic acid [74]. Biogenetically, phenolic compounds are metabolized either by the shikimic acid pathway where lignin is formed, or the malonic acid pathway, in which the major products are the simple phenol or both (shikimic and malonic), where most of flavonoids are formed [74,103]. They are symbolized by an aromatic ring with hydroxyl groups.

Phenolic compounds play an essential role in plants including growth, pigmentation, and reproduction protection against pests [103]. Several researchers have reported that consumption of phenolics can play a crucial role in human health by controlling metabolism, weight, chronic disease, and cell proliferation due to their antioxidant power and free radical scavenging properties, among other characteristics [73]. In food production, polyphenols can increase the shelf life of products by preventing oxidation due to their antioxidant activity, which relies mainly on the number and position of the hydroxyl groups related to the carboxyl functional group [73,103].

Flavonoids are one of the most well-known bioactives in the phenolics class. More than 4000 flavonoids have been discovered currently, and they are commonly present in fruits, seeds, leaves, bark, and flowers of plants. In plants, these compounds play a significant role in plant pigmentation, protecting them against pathogens [104,105]. Flavonoids are known for their antioxidant and chelating properties, which provide substantial health benefits such as the control and prevention of cancer, cardiovascular disease, and chronic inflammatory conditions [105]. To date, SFE (Table 6) has been established as an effective method to extract phenolic compounds from different plant materials.

Table 6. Examples of phenolics extracted by supercritical CO_2.

Compounds	Plant Material	Studied Parameters	Country	References
Flavonoids	*Strobilanthes crispus* (Pecah Kaca) leaves	Pressure: 100, 150 and 200 bar Temperature: 40, 50 and 60 °C Dynamic extraction time: 0, 40, 60 and 80 min	Malaysia	[104]
	Odontonema strictum leaves	Pressure: 200 and 250 bar Temperature: 55–65 °C CO_2 flow: 15 g/min Co-solvent: ethanol 95% Extraction time: 210–270 min	Burkina Faso	[106]
Flavonoids (*Tiliroside*)	*Tilia* L. flower	Pressure: 100–220 bar Temperature: 45–80 °C Time: 20–60 min	Poland	[107]
Total phenolic compounds, total flavonoids	Radish leaves	Pressure: 300 and 400 bar Temperature: 35, 40, and 50 °C CO_2 flow rate: 10 g/min Co-solvent: ethanol	Argentina	[34]
Flavonoids (hesperidin, nobiletin, and tangeretin)	Citrus genkou peels	Pressure: 100–300 bar Temperature: 40–80 °C	Japan	[108]
Total phenols	Strawberry (*Arbutus unedo* L.)	Pressure: 150, 250, 350 bar Temperature: 40, 60, 80 °C Co-solvent: ethanol (0, 10, 20%) CO_2 flow rate: 15 g/min Extraction time: 60 min	Turkey	[46]

Table 6. *Cont.*

Compounds	Plant Material	Studied Parameters	Country	References
Fatty acid	Pomegranate seed oil	Pressure: 240, 280 and 320 bar Temperature: 40,50 and 60 °C CO_2 flow rate: 133.3 g/min Extraction time: 180 min	Italy	[42]
	Yacon leaves	Pressure: 150–250 bar Temperature: 30–70 °C Co-solvent: ethanol	Brazil	[109]
Phenolic compounds	*Hibiscus sabdariffa*	Pressure: 150–350 bar Temperature: 40 to 60 °C Co-solvent: ethanol 7–15% Total flow: 25 g/min Extraction time: 90 min	Spain	[110]
Tocotrienols	Annatto seed	Pressure: 100, 170, 240 and 310 bar Temperature: 40 and 60 °C CO_2 densities: 290–915 kg/m^3	Brazil	[65]
Tocopherol	Quinoa (*Chenopodium quinoa* Will)	Pressure: 200–400 bar Temperature: 40–60 °C	Spain	[111]

From the studies listed in Table 6 regarding phenolic extraction with SC-CO_2, the variables to be taken into consideration can differ due to several factors such as the plant material, targeted compounds, and working conditions. However, the optimal conditions were considered a pressure of 200 bar, temperature of 60 °C, and 5% ethanol as the co-solvent.

6. Current Applications of Supercritical Fluid Extraction

Supercritical fluid-based technologies are involved in a broad spectrum of industrial applications that have experienced significant progress in recent years. Extraction with supercritical fluids has been applied extensively in the food, pharmaceuticals, and cosmetics industries.

6.1. Application of SC-CO_2 in Food Industry

One of the primary trends in the food industry is the demand for all-natural food ingredients free of chemical additives. Natural food antioxidants are derivatives of plant by-products. Food waste valorization is under intense worldwide investigation topic and supercritical fluid extraction has been reported as one of the best ways to valorize agro-industry byproducts. Those extracts showed sustainable use in food industries as a good source of phenolic compounds with antioxidant activities [93,112–115]. For example, the extract of mango peel has been used as a natural antioxidant in sunflower oil to control lipid oxidation; the optimal conditions were 25 MPa, 60 °C and 15% ethanol in water by using a Box–Behnken design [92]. Supercritical fluid extraction is also applied in the extraction of cholesterol and other lipids from egg yolk [116], extraction of lipids and cholesterol from meat and meat products [117], decaffeination of coffee and tea [24,118,119], extraction of hops [71,120,121], extraction of bioactive compounds [65,91,101,102], extraction of free amino acids [122], extraction of lipids and cholesterol from fish [123], extraction of natural glycosides [124], fractionation of natural colorings, natural flavorings, and fragrances from several foodstuffs [99,125], and the separation of spices and essential oils [82–86].

6.2. Application of SC-CO_2 in the Pharmaceuticals Industry

In the pharmaceuticals industry, development in the properties of active pharmaceutical ingredients is generally preferred. A significant challenge in this respect is particle size reduction, which increases bioavailability [126]. The primary use of supercritical fluids in the pharmaceuticals industry involves processes such as particle formation for drug delivery systems and crystal engineering; complexing cyclodextrins; coating, foaming and tissue engineering; impregnation [127] and purification of pharmaceutical excipients; and sterilization and solvent removal.

The best application of supercritical fluids in pharmaceuticals deals with the isolation of bioactive compounds from a mixture (purification from reactions, quantification of an active enantiomer, extraction from a natural matrix) [24]. Different plants have been used for the extraction of compounds to be used in pharmacy, such as *Catharanthus roseus*, a rich source of alkaloids, from which two dimeric alkaloids were extracted that are extensively used as antineoplastic drugs vinblastine and vincristine [79,126]. *Artemisia annua* L. exhibits a vigorous antimalarial activity due to the existence of artemisinin and its derivatives (like dehydroartemisinin). These compounds are particularly active against drug-resistant strains of *Plasmodium falciparum* [72]. *Melocactus zehntneri* is a medicinal cactus that is unusual pharmacologically because it contains isoquinoline and indole alkaloids, which are used in several drugs [128].

6.3. Application of SC-CO_2 in the Cosmetics Industry

Cosmetics are daily use products that are currently available in a different market. Nowadays, people are interested in natural products, and they have realized that one of the most active ingredients of cosmetics is the antioxidant, which can interrupt radical-chain processes, help skin repair systems, improve cell rejuvenation and prevent skin-cancer [129]. Different research has shown that SC-CO_2 extraction is a great technique that provides natural extracts with attractive fragrances or active ingredients that add value to the cosmetics by enhancing their practical action and attributes. SC-CO_2 has been used for the isolation of antioxidants and parabens from cosmetic products [130]. Vogt et al. [131] investigated the extract of blackcurrant seeds, strawberry seeds, hop cones, and mint leaves obtained by SC-CO_2 for the production of shower gels and shampoo, and the result showed that those natural extracts could be used as ingredients in shower gels.

7. Conclusions

The literature reviewed herein has shown that SFE is a separation technique commonly employed to extract natural bio-active compounds from plants due to the unique properties of supercritical fluids, that is, they simultaneously behave like a liquid and like a gas. The interest in supercritical fluid extraction is not only at the laboratory level as an analytical tool but also in industrial processing, mainly decaffeination of coffee or tea, extraction of essential oils, extraction of high added value compounds and fatty acids. Supercritical fluid extraction is favored due to its high selectivity, high efficiency, and short extraction time. Thus, in this review, factors influencing the supercritical fluid extraction process (temperature, pressure, and co-solvent) were discussed using examples. We assume that this technology will experience continued growth in the coming years and will be beneficial to sustainable development, given its green credentials, and will help to reduce the use of organic chemicals.

Funding: This research was funded by the Polish National Science Centre, grant number 2018/31/B/NZ9/03485.

Conflicts of Interest: The authors declare no conflict of interest.

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Statement about the contribution of authors

Publication P2

Uwineza, P.A.; Gramza-Michałowska, A.; Bryła, M.; Waśkiewicz, A. 2021. Antioxidant Activity and Bioactive Compounds of *Lamium Album* Flower Extracts Obtained by Supercritical Fluid Extraction. *Appl. Sci.*, 11, 7419. https://doi.org/10.3390/app11167419.

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MDPI

Article

Antioxidant Activity and Bioactive Compounds of *Lamium album* Flower Extracts Obtained by Supercritical Fluid Extraction

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Abstract: In this research, supercritical CO_2 extraction is applied to extract bioactive compounds from *Lamium album* (white dead nettle, *Lamiaceae*). Extraction was performed at various temperatures (40, 50, and 60 °C) using methanol as co-solvent at a constant flow rate of CO_2, methanol, and pressure. The collected extracts were characterized in terms of antioxidant capacity by using DPPH, ABTS and FRAP in vitro antioxidant activity assays, whereas the Folin–Ciocalteu procedure was employed to estimate the total phenols content (TPC). On the other hand, phenolic compounds in the extracts were quantitated by liquid chromatography coupled with a photodiode array detector (UPLC-PDA) and confirmed with a mass detector (TQD). The extracts have shown high TPC ranged between 234.17 to 650.17 mg GAE/g extract. DPPH scavenging of the extracts was estimated and obtained EC50 values ranged from 0.12 to 0.37 mg/mL of solution. The ABTS radical scavenging activity ranged from 43.20 to 44.53 µg TE/g. The FRAP value was found within the range of 19.48 to 44.74 µmol TE/g of extract. Differences between extraction conditions were observed. In this research, 50 °C/250 bar was efficient for the TPC, DPPH, ABTS, and FRAP assays; moreover, statistically, TPCs and FRAP assay showed significant differences between the conditions at $\alpha = 0.05$. The identification of phenolic compounds in the obtained extract of *Lamium album* flowers, using UPLC/PDA, revealed that chrysin, pinostrobin, myricetin, and *trans*-3-hydroxycinnamic acid were the significant molecules present, which may be responsible for the high content of polyphenols and antioxidant activity. The results obtained indicated that SC-CO_2 could be considered an alternative method for extracting bioactive compounds of *Lamium album*. High antioxidant activity and the presence of various bioactive compounds indicate the potential of this plant from the *Lamiaceae* family and the possibility of its application in various industries, including agriculture, food technology, or pharmacy.

Keywords: white dead nettle; polyphenols; bioactive compounds; antioxidant assays; TPC; DPPH; ABTS; FRAP; supercritical fluid extraction; UPLC/PDA

Citation: Uwineza, P.A.; Gramza-Michałowska, A.; Bryła, M.; Waśkiewicz, A. Antioxidant Activity and Bioactive Compounds of *Lamium album* Flower Extracts Obtained by Supercritical Fluid Extraction. *Appl. Sci.* **2021**, *11*, 7419. https://doi.org/10.3390/app11167419

Academic Editor: Nina Kashchenko

Received: 20 July 2021
Accepted: 9 August 2021
Published: 12 August 2021

1. Introduction

Plants have been used by humans as both food and medicine since prehistoric times. Currently, there is an interest in the use of bioactive compounds, especially polyphenols, derived from plant materials in food preservation, pharmaceuticals, cosmetics, and natural pesticides. These bioactive compounds can play a preventive or supportive role in the treatment of various metabolic disorders and diseases due to their antioxidant, antimicrobial, anticancer, and anti-inflammatory properties [1]. *Lamium album*, widely known as non-sting nettle or white dead nettle, is a perennial herbaceous plant with white blooms that is inherent to Europe, Western Asia, and North Africa [2]. It belongs to the *Lamiaceae* family, which is recognized for producing plant species high in different bioactive compounds that generally differ in structures, biological properties, and mechanisms of action.

Furthermore, the genus *Lamium* contains nearly 40 species; among them, the *Lamium album* is the most widely distributed species [3]. It has a wide variety of biological activities due to its various phytochemicals such as iridoids, flavonoids, triterpenes, fatty acids, polysaccharides, saponines, phytoecdysteroids, phenolic acids, amines, essential oils, tannins, and mucilage [4]. Flowers and leaves of the Lamium album are considered food for humankind [2]. They can be eaten raw or cooked. It is also used in teas, dietary supplement products, and its intake is primarily connected with its health benefits [2,5]. The dried flowers of this plant have demonstrated uterotonic, astringent, antispasmodic, and anti-inflammatory activities and consequently are used in menorrhagia, uterine hemorrhage, vaginal and cervical inflammation, and leukorrhea treatment [3,6]. Additionally, *L. album* flowers have been reported to possess antioxidant, free radical scavenging, and antiproliferative properties [3]. Nowadays, the beneficial understanding of natural products has been substantially increased and scientifically proven, allowing a better grasp of their valuable features. Therefore, a significant quantity of information regarding the efficacy of *L. album* extracts and raw material has been accumulated, and several health-related benefits have been successfully verified. Various studies in vivo or in vitro of *Lamium album* extracts (methanol, ethyl acetate and heptane, butanol, ethanol, and chloroform) have been done, and their antioxidant, cytoprotective, anticancer, antiviral, antimicrobial, and antiproliferative activities have been reported [5,7–9]. Additionally, bioactive phenolic compounds of this plant were also studied, and the results have shown that the crucial compounds mainly include; phenylpropanoid verbascoside, isoscutellarein derivatives, and isoverbascoside, some phenolic acids such as caffeic acid, ferulic acid, syryngic acid, and flavonoids [4,5]. Various conclusions from the mentioned studies have shown a possible correlation between phenolic compounds and the antioxidant activity of this plant and its natural health benefits [8].

Despite all of the positive attributes claimed to the *Lamium album*, there are very few publications on *L. album,* and the data are incomplete. It is vital to carefully study the properties of this plant using modern techniques, including advanced techniques for bioactive extraction such as ultrasound, pulsed electric field, extrusion, microwave heating, ohmic heating, supercritical fluids, and accelerated solvents as they improve the quantity, quality, and selectivity of bioactive components extracted from plant matrix [10] and advanced analytical techniques for identifying and quantitating different bioactive components such as ultrahigh-performance liquid chromatography with photodiode array or/and mass spectrometer (UPLC/PDA or LC/MS/MS), gas chromatography, etc., which have been claimed to be cost-effective, repeatable, accurate, linear, precise, and resilient. However, no unique extraction method is recognized to be the best one for extracting bioactive compounds from plants due to their diversity. Therefore, to optimize the qualitative and quantitative properties of the bioactive compounds, the selection of an appropriate extraction technique and conditions are critical [11].

Supercritical fluid extraction (SFE) is an advanced and environmentally friendly technique used to extract bioactive compounds from different materials. Supercritical CO_2 (SC-CO_2) is one of the most often used supercritical solvents due to its good features, including non-toxic, cheap, non-explosive, and mild critical conditions (critical temperature 31.7 °C and critical pressure approximately 73.8 bar) [12]. Moreover, SC-CO_2 has physicochemical qualities that are intermediate between those of a liquid and a gas, with low viscosity, high density, and diffusivity that allow it to be an excellent alternative to traditional extraction technologies [13]. This technique can be used for a broad spectrum of chemical and biochemical extraction processes [14] because of its selectivity property, which can be adjusted by minor changes in pressure and/or temperature. SC-CO_2 produces excellent extracts with yields that are equivalent to those made using organic solvent extraction. Some results have shown the highest antioxidant and antifungal properties of SC-CO_2 extract compared to the conventional method [15,16]. In addition to these, CO_2 has the benefit of being appropriate for extracting thermo-labile chemicals since it does not require extensive solvent heating. However, one of the limitations of this technique is

that CO_2 is a non-polar solvent and may not be effective alone to extract polar compounds such as phenolics. As a result, there is an application of methanol, water, or ethanol as co-solvent or modifier to enhance the solvation power of SC-CO_2, improve its affinity for poorly soluble solutes (alkaloids, phenolics, and glycosidic compounds), increase solubility, as well as the extraction yield with respect to operating pressure and temperature [13].

The basic goals of this research were to extract the dried *Lamium album* flower by using SC-CO_2 with methanol as co-solvent, to investigate in vitro antioxidant capacity of these extracts using different radical scavenging assays (DPPH and ABTS), reducing power assay (FRAP), total phenols estimation using Folin–Ciocalteu, and phenolic compounds quantitation by using UPLC-PDA (Waters, Manchester, MA, USA). This approach should complement the previously known extraction techniques of the *Lamium album* and improve the dissemination of this green technology and the possibility of using *Lamium album* flower extract in agriculture, food, cosmetics, and pharmaceutical industries.

2. Materials and Methods

2.1. Plant Material

High-quality of dried *Lamium album* flowers were collected from ecological area (Podlasie) in Poland and certified by herbal company Dary Natury.

2.2. Chemicals

Carbon dioxide (CO_2, SFE grade), contained in a dip tube cylinder, was purchased from Air Products Sp, Poland. Methanol for HPLC-super gradient were purchased from POCh (Gliwice, Poland), Folin–Ciocalteu's reagent, and hydrochloric acid 35–38% purity was purchased from Chempur (Piekary Śląskie, Poland). Glacial acetic acid, 3,4,5-trihydroxybenzoic acid (gallic acid), and 2,2-dyphenyl-1-picrylhydrazyl (DPPH), iron (III) chloride, sodium acetate, sodium carbonate anhydrous, potassium acetate, phosphate buffered saline tablet, potassium persulphate, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), and 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All phenolic standards including *trans*-ferulic acid, *trans*-cinnamic acid, *trans*-hydroxycinnamic acid, syringic acid, synapic acid, vanillic acid, *p*-coumaric acid, caffeic acid, caffeic acid phenethyl ester (CAPE), kaempherol, quercetin, (+)-catechin, (−)-epicatechin, apigenin, rutin, myricetin, pinocenbrin, pinobanksin, pinostrobin, galangin, chrysin, and naringenin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of analytical grade.

2.3. Supercritical Carbon Dioxide (SC-CO_2) Extraction

The extraction process was carried out on a laboratory scale by using MV-10ASFE (Waters, Manchester, MA, USA) consisted of a CO_2 cylinder and a cooling system, fluid delivery module, column oven, back pressure regulator, heat exchanger and fraction collection module with ChromScope v1.20 software (Waters, Manchester, MA, USA). Then, 2 g of *Lamium album* flower was kept in the extraction vessel of 25 mL and placed in the oven set at the desired different temperatures (40, 50 and 60 °C) and pressure (250 bar). The CO_2 flow rate was 4 mL/min, and methanol was fixed at 1 mL/min. Once the set temperature and pressure (at solvent pumps and back pressure regulator) were achieved after turning on the injection valve and the system was in equilibrium, the extraction was carried out for 180 min in each experimental run, which was composed (based on the trial experiments) of 1st dynamic time 45 min, static time 15 min and 2nd dynamic time of 120 min. *Lamium album* extracts were collected in flasks placed in a fraction collection module, and it was stored at −20 °C before further analysis of polyphenols and antioxidant activity.

2.4. Quantification of Total Phenolic Compounds

Total phenolic content (TPC) of *Lamium album* extract was estimated spectrophotometrically according to the Folin–Ciocalteu method by following the protocol of Arabshahi-

Delouee and Urooj (2007) [17] with minor modifications. Gallic acid (50–500 mg/mL) [18] was used for the standard calibration curve and distilled water as a blank sample. In a test tube, 1.60 mL of distilled water were mixed with 20 µL of samples, blank or standard to be analyzed. 100 µL of Folin–Ciocalteu reagent was then directly added and vortexed. After 3 min, 300 µL of 75 g/L Na_2CO_3 was added and mixed toughly. The obtained solution was incubated at room temperature for 45 min in absence of light after which absorbance readings were taken at 760 nm using a Varian Cary 300bio UV-VIS spectrophotometer (Agilent, Santa Clara, CA, USA). All measurements were performed at least in triplicate and the total phenolic content estimation was calculated by using this formula according to Mabrouki et al. (2018) [19]:

$$TPC = (c \times v)/m \quad (1)$$

where c is the concentration of gallic acid established from the calibration curve (mg/mL), v is the volume of extract solution (mL) and m is the weight of the sample extract (g). the results were expressed as mg gallic acid equivalent per gram of the extract (mg GAE/g of extract).

2.5. Evaluation of Antioxidant Activity

2.5.1. DPPH Assay

2,2-Diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging capacity assay is a decolorization assay that directly measures the scavenging capacity of antioxidants with DPPH radicals by monitoring its absorbance at 517 nm with a spectrophotometer [20].

The free radical scavenging activity of *Lamium album* extract was measured by DPPH assay according to the method described by Moradi et al. (2016) [21] with minor modifications. The working solution was prepared by making a methanolic solution of DPPH (0.1 mM). A 2850 µL of this solution was mixed with 150 µL of the sample, standard (Trolox) at different concentrations or blank (methanol). The reaction mixture was shaken well and incubated in the dark for 30 min at room temperature. After this time, the absorbance was taken at 517 nm using Varian Cary 300bio UV-VIS spectrophotometer (Agilent, Santa Clara, CA, USA). Low absorbance of the reaction mixture indicated high free radical scavenging activity. The experiment was carried out in triplicate, and the results are average values. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

$$DPPH^{\bullet} \text{ inhibition } \% = (Ab - As)/Ab \times 100 \quad (2)$$

where Ab is the absorbance of the blank and As is the absorbance of the sample. The EC50 value, the amount of antioxidant necessary to halve the initial DPPH concentration, was estimated using linear regression of plots of DPPH radical inhibition percentages versus the concentrations of the tested extracts of *Lamium album* flowers. The results were used for comparing the quality of the antioxidant extracts. The experiment was repeated three times at each concentration.

2.5.2. ABTS Assay

The assay estimates the capacity of antioxidants to scavenge the stable radical cation $ABTS^{+}$ (2,2′-azinobis (3- ethylbenzothiazoline-6-sulphonic acid). Antioxidant activity was determined with the modified method proposed by Re et al., 1999 [22], where the blue-green color of $ABTS^{+\bullet}$ was decreased in the presence of antioxidants. The $ABTS^{+}$ radical was prepared by mixing equal amounts of two stock solutions (7 mM ABTS solution and 2.45 mM potassium persulfate solution) and allowing them to react for 16 h at room temperature in the dark. The working solution was then prepared by mixing 3.9 mL of $ABTS^{+}$ with 140 mL of 5 mM phosphate buffered-saline (pH 7.4) to obtain an absorbance of (0.70 ± 0.02) at 734 nm using the spectrophotometer. Fresh $ABTS^{+\bullet}$ working solution was prepared daily. Then, 2850 µL of $ABTS^{+}$ working solution were allowed to react with 150 µL of plant extracts in a test tube for 8 min in water bath incubation at 30 °C. The

absorbance was measured at 734 nm using a Varian Cary 300bio UV-VIS spectrophotometer (Agilent, Santa Clara, CA, USA). Trolox was used as standard, PBS as the blank. The results are expressed in µg Trolox equivalents/g of extract mass (µg(TE)/g) on the basis of the calibration curve according to the following formula:

$$\text{ABTS value (µg TE/g of extract)} = (c \times v)/m \quad (3)$$

where c is the Trolox concentration (µg/mL) of the corresponding standard curve of the plant extract, v is the sample volume (mL), and m is the weight of the plant extract (g) [23].

2.5.3. Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing power of *Lamium album* extract was determined using a modified version of the FRAP assay [24]. This method is based on the reduction of a colored pale yellow ferric complex (Fe^{3+}-tripyridyl triazine) to a blue-colored ferrous complex (Fe^{2+}-tripyridyl triazine) by the action of electron-donating antioxidants at low pH. The working FRAP reagent was prepared daily by mixing 10 mL of 300 mM acetate buffer of 3.6 pH, with 1 mL of 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) in 40 mM hydrochloric acid and with 1 mL of 20 mM ferric chloride. The reaction mixture was incubated for 15 min at 37 °C in a water bath before used. Then, 3 mL of freshly prepared FRAP reagent was added to 100 µL of sample solutions or standard (Trolox) and incubated for 5 min at 37 °C in a water bath before analysis. Then, the absorbance of the samples was measured at 593 nm by using Varian Cary 300bio UV-VIS spectrophotometer (Agilent, Santa Clara, CA, USA). All measurements were carried out in triplicate. The results were expressed as the FRAP value calculated into µmol/L Trolox on the calibration curve, which was prepared using a methanolic solution of Trolox at the concentration 150–3000 µmol/L. The following formula was used:

$$\text{FRAP value} = (AS/AC) \times 2 \text{ concentration of standard (µmol/L)} \quad (4)$$

where AS is absorbance of the sample and AC is the absorbance of the standard (Trolox) at 593 nm and concentration of Trolox (µmol/L). From the obtained FRAP value, the final antioxidant activity in each sample was expressed as Trolox equivalents (TE) in terms of µmol TE/g extract, and the following equation according to Tomasina et al. (2012) [25] was employed:

$$\text{AA (µmol TE/g extract)} = \text{Trolox (µmol/L)/sample (g/L)} \quad (5)$$

2.6. Quantitative Analysis of Polyphenols Using UPLC-PDA

The concentrations of bioactive compounds in the methanolic extracts were determined using a UPLC-PDA-TQD system, which consisted of the Acquity UPLC chromatograph (Waters, Manchester, MA, USA) coupled with a photodiode detector (PDA eλ Detector) (Waters, Manchester, MA, USA) and an electrospray ionization (ESI) triple quadrupole mass spectrometer (TQD) (Waters, Manchester, MA, USA). Mobile phase consisted of two solutions: line A—water containing 0.1% HCOOH and line B methanol containing 0.1% HCOOH. The phenolics were separated at 30 °C on the analytical column—a Waters ACQUITY UPLC BEH C18 (150 × 2.1 mm/ID, with 1.7 µm particle size) (Waters, Manchester, MA, USA) with flow rate 0.35 mL/min and using gradient elution: from 10 to 60% of B line during 15 min followed by the return to the initial conditions. Temperature of samples in the autosampler was 10 °C. UV spectral data for all peaks were recorded in the range of 190–450 nm. Data processing was done using Empower 3 (Waters, Manchester, MA, USA). All samples of *Lamium album* extracts were filtered through a 0.20 µm syringe filter (Chromafil, Macherey-Nagel, Duren, Germany) before analyses and were injected to the chromagraphic system in triplicate. Identification of bioactive compounds was done using matching retention times, UV and mass spectra (MRM) data of standards.

2.7. Statistical Analysis

The experimental data were statistically evaluated using the Statgraphics 4.1 software package (Graphics Software System, STCC, Inc., Rockville, MD, USA). A one-way ANOVA was used to assess the significance of the differences between the antioxidant activity and polyphenols concentration in the tested extracts. Fisher's Least Significant Difference (LSD) test at $\alpha = 0.05$ was used for the paired tests.

3. Results and Discussion

3.1. Total Phenolic Content

The use of an environmentally friendly, modern extraction technique (SFE) allowed obtaining pure fractions collected at different temperatures. Supercritical CO_2 is a non-polar solvent, but its polarity can be modified by temperature and pressure variations. Polyphenols with high molecular mass are hardly soluble in pure CO_2, but their solubility can be enhanced by adding a polar co-solvent or increasing pressure [26]. In our research, methanol commonly used for extraction and high pressure (250 bar) were used. Testing the antioxidant activity allowed us to check the differences between fractions obtained at different temperatures. The TPCs of the extracts obtained from dry *Lamium album* flower by SC-CO_2 with methanol as co-solvent were determined quantitatively using a spectrophotometric method with Folin–Ciocalteu reagent as the most and commonly used method for total phenols estimation. A calibration curve was established with gallic acid as standard at different concentrations. The results were expressed in mg of gallic acid equivalent (GAE)/g of extract. All measurements were taken in triplicate, and mean values were calculated. The results of determination of the polyphenols are presented in Table 1.

Table 1. Total phenolic content of *Lamium album* flower for extracts obtained at different variants of extraction.

Extraction Conditions	TPC (mg GAE/g of Extract)
40 °C	234.17 ^{a} ± 2.48
50 °C	650.17 ^{b} ± 3.86
60 °C	418.50 ^{c} ± 2.92

Values are mean ± s.d., $n = 3$, values with the same lowercase letters in the same column indicate no significant difference at level of 5% ($p < 0.05$).

As shown in Table 1, the results varied from 234.17 to 650.17 mg GAE/g of extract for the studied conditions, which represent a variation of approximately three folds. The extract at 50 °C showed the highest TPCs of 650.17 mg GAE/g of extract, followed by 60 °C, which showed 418.50 mg GAE/g of extract, and 40 °C showed the lowest value of 234.17 mg GAE/g of extract. All studied conditions showed a considerable amount of total phenols and they were significantly different as expected due to the effect of temperature on the density and selectivity of the solvent during SC-CO_2. However, a degradation effect may be suspected between 50 °C and 60 °C at 250 bar during the extraction process, which has caused a decrease in TPCs.

The total phenolic content obtained in this work is not directly comparable to other studies of *Lamium album* extracts because the extraction methods are different. In the previously published papers, most of them have used solvent extraction methods to extract *Lamium album*. Matkowski and Piotrowska (2006) [27] reported 192 mg GAE/g of extract of TPC in methanolic extract of *Lamium album*, Pereira et al. (2012) [5] reported 500.7 ± 50.0 mg/g of extract of phenolic compounds in the ethanolic extract of *L. album* [5], also other species of *Lamium* have been reported to have high content of polyphenols [7,27,28] and methanol have been reported as a good solvent [28]. The high TPC value obtained for our extract (especially for the fraction at 50 °C) compared to the research of other authors indicates a very effective extraction using the SFE technique.

3.2. Antioxidant Activity Determination

An antioxidant is any chemical that, when present at lower concentrations than those of an oxidizable substrate, considerably slows or inhibits its oxidation. Natural antioxidants and their health advantages have received a lot of attention in recent years. Antioxidant-based medication compositions are used to prevent and cure a wide range of diseases and ailments. Plants are a significant source of natural antioxidants; they produce a diverse spectrum of secondary metabolites with antioxidative activity and therapeutic potential [29]. The antioxidative activity are mostly based on two reaction mechanisms such as hydrogen atom transfer (HAT) and single electron transfer (SET) [30].

Currently, there is no unique method that has been standardized for antioxidant activity determination due to the various reaction features, mechanisms as well as variable phase localizations that are frequently involved in the process [30]; however, while extracting valuable bioactive compounds from various plants, it is very important to select the appropriate method and optimize the extraction conditions, since this has a direct impact on the production of extracts with high activity. In this study, the assessment of antioxidant activity of *Lamium album* flower extract was conducted by two free radical-scavenging methods, the DPPH free radical scavenging assay and the ABTS radical cation decolorization assay; and by ferric reducing antioxidant power assay (FRAP) in order to have a better estimation on the antioxidant capacity of the *Lamium album* extract. These assays were conducted using a UV-VIS spectrophotometer.

The antioxidant activity of the sample extracts was examined, and the findings were slightly different. However, all of the assays could be used to determine the antioxidant activity of *Lamium album* flower extract obtained by SC-CO_2 with methanol as a co-solvent because they all yielded similar rankings among the extracts and demonstrated that extracting the antioxidants at 50 °C and 250 bar was the best condition in this study. The results are summarized in Table 2.

Table 2. Antioxidant activities estimated by DPPH, ABTS and FRAP assays of extracts from dry *Lamium album* flowers extracted using SC-CO_2 and methanol as co-solvent.

Extraction Conditions	DPPH EC50 (mg/mL)	ABTS (µg TE/g)	FRAP (µmol TE/g of Extract)
40 °C	$0.37^{a} \pm 0.04$	$43.20^{a} \pm 0.20$	$19.48^{a} \pm 0.05$
50 °C	$0.12^{b} \pm 0.00$	$44.53^{b} \pm 0.02$	$44.74^{b} \pm 0.07$
60 °C	$0.16^{b} \pm 0.00$	$44.52^{b} \pm 0.02$	$28.32^{c} \pm 0.12$

Values are mean ± s.d., $n = 3$, values with the same lowercase letters in the same column indicate no significant difference at level of 5% ($p < 0.05$).

3.2.1. DPPH Scavenging

This approach has been introduced for the 1st time by Blois (1958) [31], and now is one of the commonly used to assess the free radical scavenging activity of various antioxidant compounds due to its accuracy [32] and it is known to give reliable information concerning the antioxidant ability of the tested compounds. It is based on the reduction of DPPH in alcoholic solutions (methanol or ethanol) in the presence of a hydrogen-donating antioxidant. The dark violet color of the DPPH radical becomes yellowish or colorless due to the antioxidant compound, causing a decrease in the absorbance at 517 nm [33,34]. Table 2 showed that the DPPH EC50 results varied from 0.12 to 0.37 mg/mL, which is the quantity of antioxidant required to reduce the initial DPPH radical concentration by 50% [20]. The lower the value, the higher the antioxidant activity. The overall results showed that the extract obtained at 50 °C had the highest ability to scavenge the DPPH radical. However, the conditions 50 °C and 60 °C were not significantly different at p-value = 0.05.

In similar studies, Paduch et al. (2007) [8] with solvents extraction, has reported free radical scavenging activity of methanol and ethyl acetate extracts of *Lamium album*, compared to Trolox and their results showed that methanol extract had free radical scav-

enging properties and an average EC50 of 0.4659 ± 4.4 mg/mL while ethyl acetate extracts did not exhibit DPPH radical reduction activity [8]. Additionally, Bubueanu et al. (2013) demonstrated DPPH free radical scavenging activity of butanolic extract of *Lamium album* in EC50 value to be 19.29 mg/mL [7].

Furthermore, the antioxidant tests were performed using the scavenging activity of the synthetic stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical and two oxygen radicals spontaneously produced in the cells—superoxide radicals and hydroxyl radicals, both of which are hazardous to the cell at high levels. In comparison to reference compounds like vitamin E and quercetin, the aqueous component of *L. album* hydro-alcoholic extract demonstrated modest antioxidant activity. However, among all examined aqueous fractions from 16 medicinal plants, the extract from *L. album* shown a considerable scavenging impact on DPPH radicals (EC50 = 0.28 mg/mL) [35].

3.2.2. ABTS Assay

This method was developed by Miller and Rice-Evans [36]. It is based on the scavenging ability of antioxidants to the ABTS radical and it can be used to analyze both hydrophilic and hydrophobic antioxidant capacities of extract [30]. Table 2 in this study shows that ABTS radical scavenging activities of *Lamium album* extracts varied from 43.20 to 44.53 µg TE/g. The extract at 50 °C showed the highest antioxidant capacity (44.53 µg TE/g), followed by 60 °C which showed a very similar value—44.52 µg TE/g and at 40 °C was the lowest—43.20 µg TE/g. Statistical analysis has shown a significant difference of the extract at 40 °C to the other two remainders.

3.2.3. FRAP Assay

This method has been developed by Benzie and strain (1996) [30]. It is based on the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) by an antioxidant under acidic solution to maintain iron solubility [24,30]. The ferric salt solution is a pale-yellow color, but when reduced to the ferrous form, this changes to blue, and absorbance at 593 nm increases. However, this method is not convenient to the compounds that act as radical quenching or hydrogen transfer, particularly thiols and protein, and it does not react with glucose and fructose [20,24].

Table 2 shows that the ferric reducing potential of *Lamium album* extracts varied significantly ($p = 0.05$) from 19.48 to 44.74 µmol TE/g of extract, which represents a variation of two-folds. The extract at 50 °C showed very strong ferric ion reducing activities 44.74 µmol TE/g of extract, followed by 60 °C which showed 28.32 µmol TE/g of extract and 40 °C showed the lowest value of 19.48 µmol TE/g of extract. Furthermore, the reducing power of plant extracts by FRAP assay have been reported in various studies [37–39].

In similar studies, Dudenno et al. (2009) [38] investigated antioxidant activities and the total phenolic content of 30 commonly used plants using DPPH, ABTS, FRAP, ORAC, and SOD assays. Their findings show that the ferric ion reducing activities of the 30 plant extracts tested did not differ significantly from their DPPH and ABTS scavenging activities [38]. Additionally, Katalinic et al. (2004) [39] examined the total phenolic content and total antioxidant potential in 70 medicinal plant infusions. Their results revealed significant differences in total antioxidant capacity (FRAP) between the medicinal plant infusions, with FRAP values ranging from 59 to 25,234 µmol FeII/L of infusion. Additionally, the significant linear correlation between total phenolics and FRAP of medicinal plant extracts was confirmed.

3.3. Profile of Phenolic Compounds

Phenolic compounds are a class of natural secondary metabolites highly present in a different part of plants that has gained a lot of scientific and medicinal attention because of the wide variety of beneficial properties such as antioxidant and anti-inflammatory qualities that may be beneficial in the prevention and/or treatment of cardiovascular disease, neurological diseases, cancer, and obesity [40].

As can be seen in Table 3, the present study identified 13 out of 22 analyzed phenolic compounds in *Lamium album* flower extract obtained by SC-CO_2, which comprised flavones, phenylethanoid isomers and flavanone. The mean phenolic compounds concentration varied from 0.09 (*trans*-cinnamic acid, at 40 °C) to 22.68 ng/µL (chrysin, at 50 °C). Apart from chrysin, high levels of concentration for extracts obtained at 50 °C have been reported for myricetin, pinostrobin, and *trans*-3-hydroxycinnamic acid. The concentration of the most analyzed bioactive compounds was statistically higher ($p = 0.05$) for the extract obtained at the temperature of 50 °C, especially in comparison with the extraction conditions at the lower temperature of 40 °C. On the other hand, for the extraction carried out at the highest temperature 60 °C, the level of most bioactive compounds was significantly lower compared to the extraction at 50 °C, except for ritin, quercitin and trans-cinnamic acid. The obtained results are consistent with the highest antioxidant activity for the extract obtained at 50 °C, confirmed by four methods (Tables 1 and 2).

Table 3. Quantitative analysis of phenolic compounds identified in *Lamium album* flower extract (ng/µL extract).

Phenolic Compounds	Parameters of Extraction		
	40 °C	50 °C	60 °C
	[ng/µL]		
myricetin	6.70 ^{a} ± 0.46	10.37 ^{b} ± 0.80	7.61 ^{c} ± 0.44
quercetin	1.89 ^{a} ± 0.17	2.89 ^{a} ± 0.14	1.35 ^{a} ± 0.28
rutin	0.36 ^{a} ± 0.63	2.49 ^{b} ± 0.17	2.33 ^{b} ± 0.14
CAPE	5.06 ^{a} ± 0.22	8.72 ^{b} ± 0.39	4.41 ^{c} ± 0.33
apigenin	3.25 ^{a} ± 0.28	6.10 ^{b} ± 0.14	3.97 ^{c} ± 0.19
pinocembrin	1.15 ^{a} ± 0.13	1.68 ^{b} ± 0.23	1.10 ^{a} ± 0.07
pinostrobin	8.15 ^{a} ± 0.18	13.84 ^{b} ± 0.29	7.13 ^{c} ± 0.15
galangin	5.82 ^{a} ± 0.33	7.79 ^{b} ± 0.31	5.07 ^{c} ± 0.14
chrysin	15.75 ^{a} ± 1.27	22.68 ^{b} ± 0.35	13.36 ^{c} ± 0.45
vanillic acid	1.96 ^{a} ± 0.18	6.94 ^{b} ± 0.16	4.19 ^{c} ± 0.36
syringic acid	3.17 ^{a} ± 0.22	7.81 ^{b} ± 0.39	6.20 ^{c} ± 0.24
trans-3-hydroxycinnamic acid	3.54 ^{a} ± 0.23	9.76 ^{b} ± 0.35	2.98 ^{c} ± 0.16
trans cinnamic acid	0.09 ^{a} ± 0.15	1.70 ^{b} ± 0.11	1.79 ^{b} ± 0.13
TOTAL	80.77	102.79	61.49

nd—not detected, values with the same lowercase letters in the same row indicate no significant difference at level of 5% ($p = 0.05$).

In the literature, very little information is available concerning the phenolic compounds of the studied plant (*Lamium album* flowers) and their application. However, in the previous studies, some flavonoids in forms of glycosides (rutin, isoquercitrin, tyliroside) and aglycones (quercetin) and phenolic acids (protocatechuic, chlorogenic, vanillic, caffeic, coumaric, and ferulic acids) have been found in methanol and ethyl acetate extracts of *L. album* by using HPTLC combined with densitometry and HPLC method [8]. For the first time, Pereira et al. (2012) [5] identified derivatives of unusual flavone isoscutellarein in purified ethanol extract of flowers, leaves and stems from *L. album*, and were found to represent approximately one third of the total phenolics quantified and also phenylethanoid glycosides verbascoside and iso-verbascoside have been reported [5].

To the best of our knowledge, most of those phenolic compounds were identified in *Lamium album* for the first time in the present study. Chrysin (5,7-dihydroxyflavone) is a major compound detected in *Lamium album* extract in this study. It is a flavone, naturally present in flowers of various plants, honey and some mushrooms [41], it has a lot of health importance for humans. Chrysin was reported to have enormous biological activities such as antibacterial, antidiabetic, anticancer, anti-inflammatory, and anti-aging [42,43]. Furthermore, a recent clinical trial found that using chrysin can result in body weight loss and a lower postprandial blood-glucose level [41]. In addition to its medicinal use as

an ingredient in pharmaceutical preparations, chrysin is widely utilized as a functional additive in teas, meals, and cosmetics.

Myricetin is a common plant-derived flavonoid that has also identified in the present study of *Lamium album* flower extract at high concentration. Myricetin is widely found in vegetables, fruits, nuts, berries, herbs, medicinal plants and beverages such as tea and wine [44]. This flavonoid has also been shown to have strong antioxidant capabilities as well as free radical scavenging properties. These activities appear to support a wide range of beneficial outcomes, including anti-platelet aggregation, antihypertensive, immunomodulatory, anti-inflammatory, anti-allergic, analgesic, and anticancer effects [44,45]. The rich composition of bioactive compounds of *Lamium album* extracts along with high antioxidant activity indicates the great potential of this poorly—so far—known plant.

4. Conclusions

Lamium album flowers extracts were obtained by SC-CO_2 with methanol as co-solvent. The antioxidant properties of the obtained extracts were estimated spectrophotometrically by using DPPH, ABTS, and FRAP assays. The findings of our study revealed that the obtained extracts from *L. album* flowers possess considerable antioxidant potential. The differences in antioxidant activities and total phenolic content in the extracts can be attributed to different extracting conditions, especially temperature in this study as it can have positive or negative effect on the solubility of bioactive compounds during SC-CO_2 extraction at constant pressure. The most efficient temperature was 50 °C. The phenolic composition of the extracts was assessed by UPLC/PDA and chrysin, pinostrobin, myricetin and *trans*-3-hydroxycinnamic acid were higher compared to other compounds in this study. Therefore, this work confirms that phenolic compounds and antioxidants can be effectively extracted from *Lamium album* flowers using green SFE technology. Our results indicate wide possibilities of using *Lamium album* flower extract in agriculture, food, cosmetics, and pharmaceutical industries as a source of bioactive compounds.

Author Contributions: P.A.U. and A.W. designed of the experiments; P.A.U. antioxidant activity and bioactive compounds determination; M.B. and A.W. development of analytical procedures; M.B. statistical analysis; P.A.U. manuscript preparation; M.B. and A.G.-M. reviewed the manuscript; A.W. supervision of research. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Polish National Science Centre, grant number 2018/31/B/NZ9/03485.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author, upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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Statement about the contribution of authors

Publication P3

Uwineza, P. A.; Urbaniak, M.; Stępień, Ł.; Gramza-Michałowska, A.; Waśkiewicz, A. 2023. *Lamium Album* Flower Extracts: A Novel Approach for Controlling *Fusarium* Growth and Mycotoxin Biosynthesis. *Toxins,* 11 (15), 651. https://doi.org/10.3390/toxins15110651.

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Lamium album Flower Extracts: A Novel Approach for Controlling *Fusarium* Growth and Mycotoxin Biosynthesis

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Abstract: *Lamium album* is a medicinal flowering plant that is rich in bioactive compounds with various biological properties. *Fusarium* species, known for causing significant crop losses and mycotoxin contamination, pose threats to food safety and human health. While synthetic fungicides are commonly employed for fungal management, their environmental impact prompts the ongoing development of alternative methods. This study aimed to evaluate the efficacy of *L. album* flower extracts in inhibiting the in vitro growth and biosynthesis of mycotoxins by *Fusarium culmorum* and *F. proliferatum* strains. The extracts were obtained by supercritical fluid extraction using CO_2 (SC-CO_2). The effects of various concentrations (2.5, 5, 7.5, and 10%) were assessed on a potato dextrose agar (PDA) medium using the "poisoning" technique. *L. album* flower extracts reduced mycelium growth by 0 to 30.59% for *F. culmorum* and 27.71 to 42.97% for *F. proliferatum*. Ergosterol content was reduced by up to 88.87% for *F. culmorum* and 93.17% for *F. proliferatum*. Similarly, the amounts of synthesized mycotoxins produced by both strains were also lower compared to control cultures. These findings are a preliminary phase for further in vivo tests planned to determine the fungistatic effect of *L. album* flower extracts on cereal substrates as seedlings incubated in controlled environments and under field conditions. Their phytotoxicity and biological stability, as well as the possibility of formulating a bio-preparation to protect cereals against *Fusarium* infections, will be evaluated.

Keywords: white dead nettle; supercritical fluid extraction; integrated pest management; plant extracts; phytopathogenic; anti-mycotoxigenic; ergosterol

Key Contribution: This manuscript evaluates the in vitro efficacy of *L. album* flower extracts in inhibiting the growth of *F. culmorum* and *F. proliferatum* mycelia, as well as the impact of the extracts on the biosynthesis of mycotoxins. *L. album* is a medicinal flowering plant known for its antifungal effects. *L. album* flower extracts were obtained through SC-CO_2 extraction and demonstrated to be promising antifungal agents against *F. culmorum* and *F. proliferatum*. Furthermore, the extracts significantly reduced ergosterol content and mycotoxin synthesis in both tested strains. These findings provide valuable insights for further research on in vivo tests and the potential bio-preparation of *L. album* for cereal protection against *Fusarium* infections, serving as an alternative to synthetic fungicides in integrated pest management programs.

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Citation: Uwineza, P.A.; Urbaniak, M.; Stępień, Ł.; Gramza-Michałowska, A.; Waśkiewicz, A. *Lamium album* Flower Extracts: A Novel Approach for Controlling *Fusarium* Growth and Mycotoxin Biosynthesis. *Toxins* **2023**, *15*, 651. https://doi.org/10.3390/toxins15110651

Received: 12 September 2023
Revised: 7 November 2023
Accepted: 10 November 2023
Published: 13 November 2023

1. Introduction

Plant diseases represent a significant constraint for global crop yield, and fungal infections account for 80% of these losses [1]. *Fusarium* species are significant phytopathogenic and mycotoxigenic fungi that cause devastating effects on the global market, food safety,

and farmers' livelihoods [2,3]. Members of this genus infect a variety of staple cereals, including wheat, oat, barley, and maize, causing wilting, yellowing, browning, necrosis, and deformation. These fungi use several mechanisms to infect and colonize plants, including the production of enzymes that allow them to penetrate the plant cell wall. The production of mycotoxins that damage plant cells and enhance the pathogen's ability to infect and colonize the plant is equally important [1,2]. Therefore, understanding these strategies is crucial for controlling *Fusarium* pathogens in plants.

Fusarium culmorum and *F. proliferatum* are polyphagous fungi that infect various plant species, particularly cereals, leading to diseases such as *Fusarium* head blight and *Fusarium* ear rot [4–7]. These diseases cause yield losses, reduce grain quality, and cause mycotoxin contamination, posing risks to human and animal health [7]. Mycotoxin contamination can occur at all stages of plant growth, influenced by local climate and the co-existence of multiple fungi [8–10]. Mycotoxins produced by these fungi, including deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEN), and fumonisins from B group (FB_1, FB_2, and FB_3), have detrimental effects on organisms [3,8,11,12]. They also pose challenges to food security by reducing crop yield, increasing food prices, and impacting global markets and low-income consumers [7]. Controlling these mycotoxins is essential to prevent economic losses, ensure food safety, and address import restrictions [13,14]. Therefore, research continues to explore sustainable techniques for protecting and controlling fungal pathogens (primary source of mycotoxins in food and feed).

In response to those challenges, various initiatives have been developed [2,7,8]. These efforts include establishing legal limits for mycotoxins in food and feed, promoting sustainable pesticide use, and investing in research and development to identify alternative control strategies. Among them, using plant-based products, such as natural plant extracts and essential oils, is a promising alternative for synthetic fungicides in sustainable agriculture [13]. These solutions offer multiple benefits, including environmental and human health safety, minimal risk of resistance, and compatibility with organic and biodegradable farming practices [15,16]. Plant extracts contain bioactive compounds that disrupt various metabolic processes in fungi, such as the biosynthesis of ergosterol, a crucial sterol present in fungal cell membranes that plays a vital role in fungal growth [12,15–19].

Lamium album, commonly known as white dead nettle or non-stinging nettle, is a medicinal flowering herbaceous plant native to Europe, Western Asia, and North Africa [20]. This plant's young shoots, leaves, and flowers are edible (fresh or cooked). Nowadays, *L. album* is used to make beverages such as tea and dietary supplements that claim to detoxify the organism and prevent menstrual disorders, abdominal inflammation, and musculoskeletal diseases [21]. It is known for its anti-inflammatory, astringent, anti-septic, antibiotic, and bacteriostatic properties [22]. Various in vitro and in vivo model systems were used to reveal its antiviral, antibacterial, antioxidant, anticancer, cytoprotective, wound-healing, and other significant pharmacological effects [22–27].

Czerwińska et al. (2020) have identified iridoids (lamalbid), phenolic acids/depsides (chlorogenic acid), phenylpropanoids (verbascoside), and flavonoids (rutin, quercetin malonylhexoside, tiliroside) in aqueous and ethanolic-aqueous extracts of *L. album* [24], rich in phenolic acids and flavonoids, which are the main bioactive compounds responsible for multiple biological activities. Pourmirzaee et al. (2019) have also reported other significant pharmacological effects of *L. album* extracts, further supporting their potential as a valuable source of bioactive compounds with diverse health-related benefits [28,29]. However, as far as we know, there has been limited exploration of *L. album* flower extracts as a source of natural antifungal agents in agriculture. Additionally, no studies have been conducted on the efficacy of *L. album* flower extracts against *Fusarium* pathogens.

Therefore, this study was designed to assess the in vitro effects of *L. album* flower extracts against *F. culmorum* and *F. proliferatum*, as well as their ability to suppress mycotoxin production. One of the most innovative aspects of this study is the evaluation of the antifungal effects of the aerial part of *L. album* against agriculturally important cereal pathogens.

2. Results

2.1. Inhibitory Effect of L. album Flower Extract against Fusarium culmorum and F. proliferatum

This study aimed to assess the antifungal activity of SC-CO_2 *L. album* flower extracts (2.5, 5, 7.5, and 10%) as compared to the control group (PDA without extract). After 10 days of incubation, an inhibitory effect of the studied concentrations of *L. album* flower extract was observed compared to the controls of both strains (Figures 1 and 2). During the initial days of incubation, the fungistatic effect of the extract (2.5% and 5%) on *F. culmorum* diminished as early as day 3. However, by the final day of incubation, an inhibitory effect on colony growth became evident for 7.5% and 10% concentrations. Conversely, the antifungal effects of all studied concentrations on *F. proliferatum* were initially similar during the early stages of incubation. However, by the sixth day of incubation, the inhibitory effects of *L. album* began to manifest, and by the last day of incubation (tenth), a positive effect of all concentrations was observed as compared to the control. In addition, the inhibitory effects varied across different extract concentrations for each strain (Table 1). For *F. culmorum*, there was no discernible growth inhibition at lower extract concentrations of 2.5 and 5% (0% inhibition). As the extract concentration increased to 7.5% and 10%, inhibitory activity gradually emerged, resulting in inhibition percentages of 23.53% and 30.59%, respectively. However, for the *F. proliferatum* strain, all tested extract concentrations reduced the mycelium growth compared to the control. Significant differences were observed between the 2.5% concentration and the remaining concentrations (5, 7.5, and 10%); however, the increase in the extract concentrations from 5 to 10% did not differ significantly. Similarly to *F. culmorum*, the highest growth inhibition of 42.97% was observed for *F. proliferatum* at the highest tested concentration (10%).

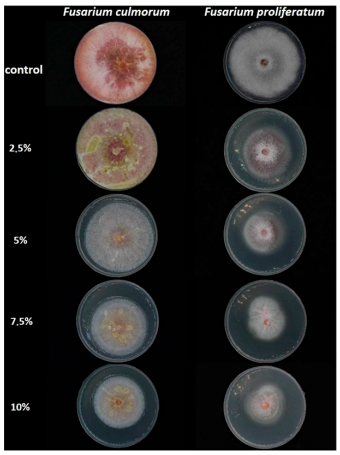

Figure 1. Petri dishes image showing the inhibitory effects of *L. album* flower extracts (2.5–10%) on mycelia growth of *F. culmorum* and *F. proliferatum* in the PDA medium after a 10-day incubation period.

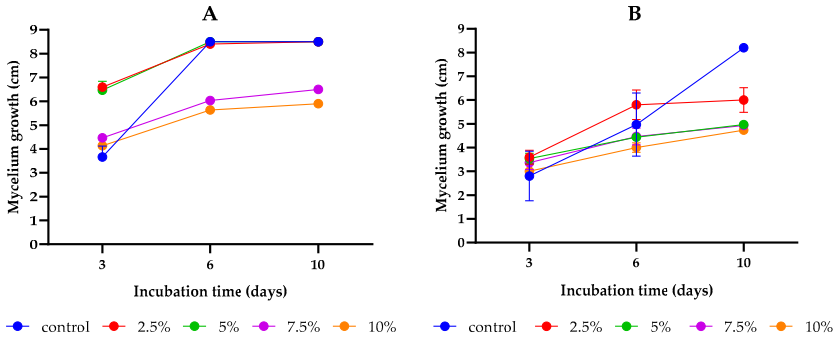

Figure 2. The impact of different concentrations (2.5, 5, 7.5, and 10%) of *L. album* on the mycelium growth of the studied *Fusarium* strains cultivated on PDA media over a 10-day incubation period: (**A**) *F. culmorum*, (**B**) *F. proliferatum*. The results are averages of three repetitions ± standard deviation.

Table 1. The inhibitory effect of *L. album* extracts (2.5, 5, 7.5, and 10% concentration) on the mycelium growth of *F. culmorum* and *F. proliferatum*.

Extract Concentration (%)	Mycelium Growth Inhibition (%)	
	F. culmorum	*F. proliferatum*
Control without extracts	-	-
2.5	0.00 ^{a}	27.71 ± 6.26 ^{b}
5	0.00 ^{a}	40.16 ± 0.70 ^{a}
7.5	23.53 ± 2.04 ^{b}	40.56 ± 0.70 ^{a}
10	30.59 ± 2.04 ^{c}	42.97 ± 0.70 ^{a}

All values are means of three replicates ± standard deviation. The superscripts of different letters in rows are significantly different (Tukey's HSD test, significant at $p < 0.01$).

2.2. The Antifungal Effects of L. album Flower Extract on the Ergosterol Content

The antifungal efficacy of *L. album* flower extract on *F. culmorum* and *F. proliferatum* growth was evaluated by measuring the ERG concentration using ultra-performance liquid chromatography with a photodiode array detector (UPLC/PDA) technique. In both strains examined, the application of *L. album* flower extracts considerably decreased the ERG content compared to the control (Table 2).

Table 2. Effects of *L. album* extracts (2.5, 5, 7.5, and 10%) on ergosterol (ERG) content (μg/g) and its reduction (%) after 10 days of incubation at 25 °C on a PDA medium inoculated with *Fusarium* species.

Extract Concentration (%)	ERG Concentration (μg/g) and Percentage of Reduction (%)			
	F. culmorum		*F. proliferatum*	
	(μg/g)	(%)	(μg/g)	(%)
Control (without extracts)	5036.19 ± 1178.93 ^{c}	-	20,234.01 ± 1484.40 ^{d}	-
2.5	3818.97 ± 829.56 ^{bc}	24.17 ^{c}	7778.33 ± 227.47 ^{c}	61.56 ^{c}
5	1864.53 ± 706.63 ^{ab}	62.98 ^{a}	4278.58 ± 281.07 ^{b}	78.85 ^{b}
7.5	1016.00 ± 200.36 ^{a}	79.83 ^{ab}	1549.54 ± 157.36 ^{a}	92.34 ^{a}
10	560.59 ± 140.09 ^{a}	88.87 ^{b}	1381.55 ± 191.88 ^{a}	93.17 ^{a}

All values are means of three replicates ± standard deviation. The superscripts of different letters in rows are significantly different (Tukey's HSD test, significant at $p < 0.01$).

The results of the UPLC/PDA analysis (Table 2) showed that when the concentration of *L. album* flower extract increased, there was a significant reduction in ergosterol production by the studied strains, i.e., there was a dose-dependent response of *F. culmorum* and *F. proliferatum* to the *L. album* extracts. At the lowest concentration tested (2.5%), the extract reduced the ergosterol content by an average of 24.17% for *F. culmorum* and 61.56% for *F. proliferatum*. As the concentration of the extracts increased to 5, 7.5, and 10%, the decrease in ergosterol became more pronounced, with the highest concentration (10%) showing the most significant inhibition of ergosterol biosynthesis, with an average of 88.87% for *F. culmorum* and 93.17% for *F. proliferatum* in relation to the control.

2.3. The Effects of L. album Flower Extract on Mycotoxin Biosynthesis

The effects of *L. album* flower extracts on mycotoxin biosynthesis were assessed using a multi-mycotoxin analysis method. The results showed that 12 different mycotoxins out of 22 examined were identified and quantified. In relation to the control samples (PDA without extracts), all tested concentrations of *L. album* flower extracts reduced mycotoxins' biosynthesis (Figures 3 and 4). The suppressive effect was variable and depended on

extract concentration and *Fusarium* isolates. Additionally, the results showed that the 10% concentration was the most effective in reducing mycotoxin biosynthesis in both strains.

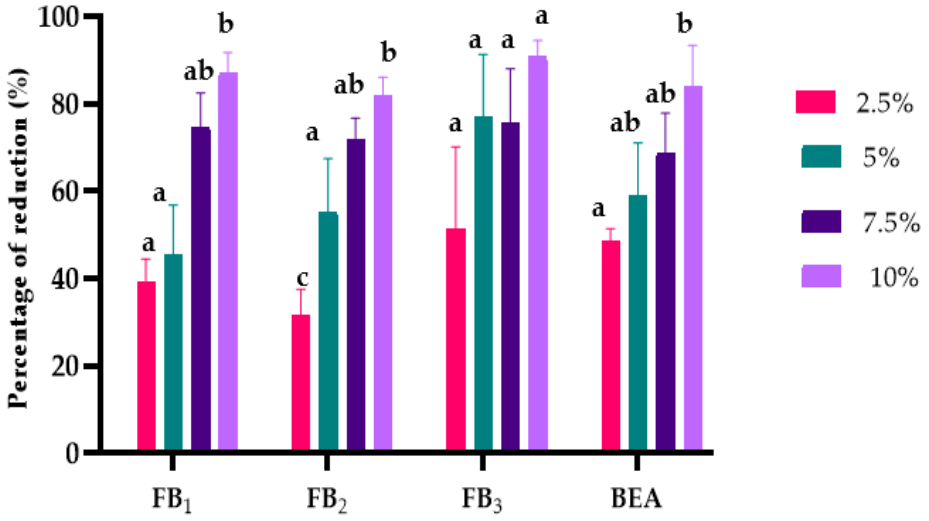

Figure 3. Effects of *L. album* extracts (2.5, 5, 7.5, and 10%) on reducing mycotoxins produced by *F. proliferatum*. The average with different letters (a–c) for each mycotoxin is significantly different at $p < 0.01$. Error bars represent standard deviation (n = 3).

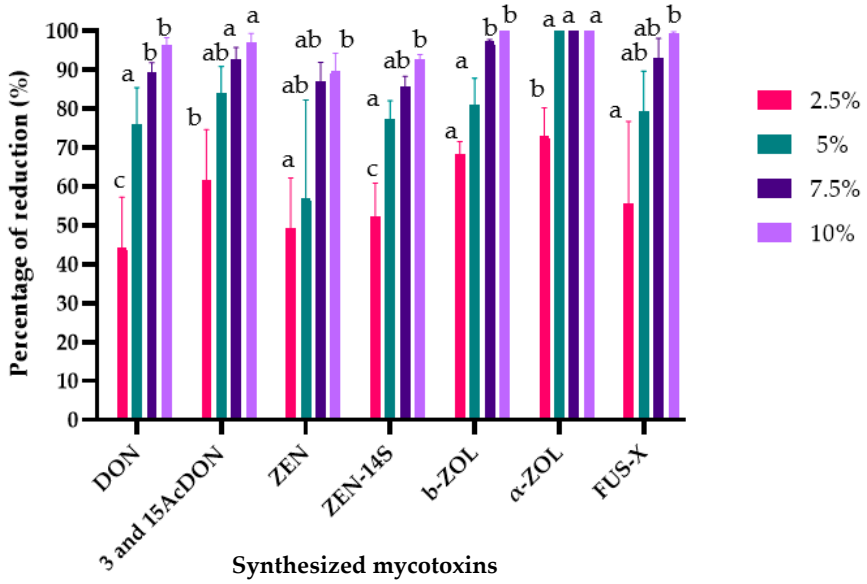

Figure 4. Effects of *L. album* extracts (2.5, 5, 7.5, and 10%) on the production of mycotoxins by *F. culmorum*. The average with different letters (a–c) for each mycotoxin is significantly different at $p < 0.01$. Error bars represent standard deviation (n = 3).

F. proliferatum synthesized FBs (FB_1, FB_2, FB_3) and beauvercin (BEA), which amounted in the treated samples to the extracts differing significantly at $p < 0.01$, except for FB_2 (Supplementary Materials Table S1). The increase in extract concentration led to a more significant reduction in mycotoxin concentration (Figure 3). Specifically, BEA was reduced by a maximum of 86.76%, FB_1 by 87%, FB_2 by 81%, and FB_3 by 90%.

Similarly, various mycotoxins, including trichothecenes (DON, 3-AcDON, and 15-AcDON) as well as ZEN and its derivatives (zearalenone-14-sulfate (ZEN-14S); β-zearalenone (β-ZOL); and α-zearalenone (α-ZOL)) and fusarenone-X (FUS-X), were produced by the inoculated *F. culmorum* strain in the treated samples and the control samples but at different concentrations (Supplementary Materials, Table S2). *L. album* flower extracts at the studied con-

centrations (2.5, 5, 7.5, and 10%) significantly reduced the mycotoxin biosynthesis (Figure 4). The reduction percentages of these mycotoxins were as follows: DON (44.39–96.41%), 3- and 15-AcDON (61.64–96.94%), ZEN (49.38–89.71%), ZEN-14S (52.33–92.61%), β-ZOL (55.57–99.86%), α-ZOL (68.42–100%), and FUS-X (55.57–99.51%).

3. Discussion

Management of *Fusarium* pathogens is one of the crucial challenges to minimizing yield loss during harvesting and post-harvest storage of various crops. *Fusarium* species are the common cause of several diseases in wheat, maize, rice, rye, and barley and lead to the accumulation of mycotoxins in grains and other plant tissues, compromising their suitability for human and animal consumption [1,4,6]. Of note is that synthetic fungicides have historically played a crucial role in combating fungi and have benefited crop protection for both small-scale and industrial farmers. However, their use is increasingly restricted or discouraged due to various concerns, including environmental impact, contamination of drinking water, and implications for human health and livestock resulting from improper or excessive use [16]. Therefore, using plant-based fungicides against fungal pathogens can help mitigate the development of resistance, primarily due to the presence of diverse antimicrobial compounds and their synergistic effects. Plant extracts are generally safe, exhibit minimal human toxicity, and are environmentally friendly [12,15]. In contrast to typical synthetic fungicides, their inherent instability at higher temperatures makes them easily biodegradable, ensuring they do not persist in the environment for extended periods [12], and antifungal activities of plant extracts towards *Fusarium* have been reported both in laboratory media and plant matrices [10,30–35].

L. album is recognized for its diverse bioactive compounds with various biological properties [10,26]. Therefore, it may be considered a natural source of fungicidal compounds.

Results of this study indicate that the *L. album* flower extracts obtained by SC-CO_2 inhibit mycelium growth and reduce ergosterol and mycotoxins biosynthesis in both *F. culmorum* and *F. proliferatum*.

Though several studies report the antifungal effectiveness of various plant extracts against *Fusarium* spp. [31–41], to the best of our knowledge, there are no existing data concerning the antifungal potential of *L. album* flower extracts against *Fusarium* spp. The observed antifungal and anti-mycotoxigenic effects of *L. album* flower extracts can be attributed to the group of bioactive compounds, including phenylpropanoids, flavonoids, iridoids, and phenolic acids, potentially present in the extracts [21–28]. These effects could arise from synergistic interactions among diverse phytochemicals found within *L. album* flowers, such as ferulic, *p*-coumaric, gallic acid, myricetin, pinostrobin, and caffeic acid, each with distinct biological properties [21,23,26,29,42]. Furthermore, one of the ongoing efforts is focused on identifying and characterizing specific compounds responsible for the observed antifungal effects of the analyzed extracts. Importantly, our prior study has characterized the antioxidant activity and phenolic compounds, including myricetin, chrysin, and trans-3-hydroxycinnamic acid, indicating that the extraction conditions employed here were optimal for obtaining bioactive compounds of high antioxidant activity [10].

Additionally, antifungal attributes of specific bioactive compounds in various plant extracts have been reported [41–44]. For instance, a study conducted by Abhishek et al. (2015) investigated the antifungal effect of *Solanum torvum* leaves against different fields and storage fungi. By sequentially extracting the leaves, researchers isolated a bioactive compound called torvoside K from the chloroform extract. Torvoside K demonstrated substantial antifungal activity against the tested fungi, as evidenced by inhibitory zones ranging from 33.4 to 87.4%. Furthermore, the compound exhibited a concentration-dependent anti-mycotoxigenic effect, ultimately reducing the presence of FB_1 in both in vitro and in vivo experiments [41]. The studies have also proven that plant extracts' antifungal potential depends not only on the main components and their concentration but also on compounds found at lower concentrations and their synergistic action. Chen et al. (2018) evaluated the antifungal activity of *Curcuma longa* extract against *F. graminearum*. Eight compounds were

identified, and all had inhibitory effects on *F. graminearum* mycelium growth. Curdione showed the highest inhibition of 52.9%, and combining curdione with the other seven compounds greatly enhanced the inhibition, reaching up to 100% [43].

The inhibitory effects of *L. album* flower extracts were concentration-dependent for *F. culmorum*, while *F. proliferatum* did not exhibit a similar trend. However, the highest antifungal activity for both strains was observed at a 10% concentration. These observations align with previous research findings that highlighted the varying impacts of natural plant extracts on fungal growth (Table 3).

Table 3. The comparative assessment of the inhibitory effect of different plant extracts against *Fusarium* spp.

Plant Material	Extraction Method	Conc *	Antifungal Assay	*Fusarium* spp.	Inhibitory (%)	Ref. *
Ruta graveolens	Solid–liquid method ethanol:water (85:15 *v*/*v*)	16 mg/mL	Agar dilution method	*F. oxysporum*	72.90	[31]
				F. proliferatum	68.73	
Azadirachta indica	Distilled water	100 mg/mL	-	*F. oxysporum*	87.40	[32]
Tithonia diversifolia					76.80	
Chromolaena odorata					62.70	
Eucalyptus camaldulensis leaves	Distilled water	4 mg/mL	Agar dilution method	*F. solani*	78.58	[33]
				F. oxysporum	77.80	
Salvia officinalis leaves	Reflux condenser (70% ethanol)	20%	Poisoned method	*F. avenaceum*	61.30	[34]
				F. culmorum	52.59	
				F. graminearum	38.39	
Solanum torvum leaves	Solvent extraction (chloroform)	1 mg/mL	Poisoned food technique	*F. verticillioides*	76.42	[41]
				F. oxysporum	68.00	

Conc * = concentration, Ref * = reference.

L. album extracts obtained by SC-CO_2 at 10% showed comparable or inferior inhibitory effects to other plant extracts across different concentrations and extraction methods, as indicated in Table 3. Reyes-Vaquero et al. (2021) showed that one plant could inhibit two different species differently (*Ruta graveolens* on *F. oxysporum* and *F. proliferatum*) [31]; similarly, Kursa et al. (2022) observed different inhibitory effects of *Salvia officinalis L.* (sage) against the three studied *Fusarium* strains [34]. This highlights the plant-specific nature of antifungal activity. Furthermore, Ngegba et al. (2018) reported that three different plants at the same concentration inhibited one fungal species differently (*Azadirachta indica*, *Tithonia diversifolia*, and *Chromolaena odorata* on *F. oxysporum*) [32]. This suggests that the effectiveness of plant extracts is influenced by the unique properties of each plant material. Therefore, the diverse antifungal effects summarized in Table 3, along with the results of our study, underscore the complexity of plant–fungal interactions, emphasizing the need for a nuanced understanding of each plant's bioactivity against specific fungal pathogens.

The assessment of ergosterol content further supported the inhibitory effects of the *L. album* extracts. The results showed a significant reduction in ergosterol content in the treated samples compared to the control group, with reductions ranging from 24.17 to 88.87% for *F. culmorum* and 61.56 to 93.17% for *F. proliferatum*, which may be explained by significant disruption of the fungal cell membrane [43]. Bodoira et al. (2020) have

shown that the extracts obtained from agro-industrial by-products of peanut, sesame, and pistachio reduced ergosterol content by 25, 66, and 33%, respectively, compared to the control [35]. However, it should be noted that the reduction in ergosterol content does not always correlate with mycotoxin reduction and vice versa [37,38]. This conflicting outcome arises from the potential unintended consequences of employing biological fungicides; instead of inhibiting fungal growth and mycotoxin biosynthesis, they may boost mycotoxin production [35,39].

A significant reduction in mycotoxins in both tested fungi was observed with increased *L. album* flower extract concentrations. These results align with previous studies on the inhibitory effects of reducing mycotoxin biosynthesis using natural plant extracts [40,41]. It is essential to highlight that these two strains synthesize distinct mycotoxins and do not share any common types, as the multi-mycotoxin analysis method showed. Heidtmann-Bemvenuti et al. (2016) investigated the antifungal activity of natural compounds, including γ-oryzanol, a phenolic extract of neem seeds and rice bran, on three toxigenic strains of *Fusarium graminearum* isolated from wheat, rice, and barley. Phenolic extracts of rice bran and neem seeds completely inhibited ZEN production and retarded NIV production. On the other hand, γ-oryzanol demonstrated a notable ability of inhibiting ZEN, achieving complete inhibition (100%) in the wheat and rice isolates. Thus, the phenolic extracts were more effective than γ-oryzanol in inhibiting the *F. graminearum* strains [40].

Among the mycotoxins produced by *F. proliferatum*, BEA exhibited the highest prominence. However, its reduction was similar to the reduction observed for other mycotoxins in this study. Similar effects have been reported for natural phenolic compounds such as ferulic acid, sinapic acid, and bromelain, which completely prevented the synthesis of BEA in *F. proliferatum* and reduced it in *F. ananatum* strains [45]. Current studies indicate a high prevalence of BEA in grains and wheat-based products such as pasta, infant formulas, breakfast cereals, and biscuits, with incidence rates ranging from 40 to 90% [46]. However, major food regulatory bodies like the US Food and Drug Administration and the European Food Safety Authority have not yet established specific regulations or guidelines for permissible levels of emerging mycotoxins, including BEA [47].

The *F. culmorum* strain produced different mycotoxins. Among them, ZEN-14S was the most prominent mycotoxin produced by this strain. ZEN-14S is a modified form of ZEN synthesized by different *Fusarium* species [48]. The results revealed that its reduction aligned with that of the other mycotoxins. Previous research has suggested the possibility of its transformation into free forms [49], a phenomenon not observed in our study. Therefore, the identification and characterization of a diverse set of mycotoxins underscore the importance of analyzing all possible mycotoxins produced by tested strains to avoid an incorrect assessment of the mycotoxin content, which poses potential health risks to consumers [13]. This study demonstrated that *L. album* flower extracts can inhibit not only well-known mycotoxins but also their modified forms and derivatives.

4. Conclusions

Applying plant extracts as a source of natural compounds against phytopathogenic and mycotoxigenic fungi presents an interesting approach, primarily as the world seeks sustainable solutions for environmental problems and food safety. The conducted experiments revealed promising in vitro effects of *L. album* flower extracts against *F. culmorum* and *F. proliferatum,* which are significant pathogens affecting cereals. The extracts inhibited the growth of these fungi significantly better at a higher concentration (10%) compared to lower concentrations (2.5, 5, and 7.5%). These inhibitory effects were further confirmed by reduced amounts of ergosterol and mycotoxins. The overall effects of *L. album* flower extracts obtained by SC-CO_2 depended on the extract's concentration, type of strain, and the biosynthesized mycotoxins. These findings offer valuable insights for future research endeavors to characterize the primary active components in the extracts derived from *L. album* flowers. Additionally, a comprehensive in vivo study will be essential to practically evaluate the effectiveness of *L. album* flower extracts against *Fusarium* pathogens

in cereal crops and to gain insight into their inhibitory mechanisms. This ongoing effort holds significant promise for promoting sustainable agricultural practices, addressing the challenges related to fungal diseases in cereal crops, and contributing to the positive enhancement of food safety.

5. Materials and Methods

5.1. Chemicals

Carbon dioxide (CO_2, SFE grade), contained in a dip tube cylinder, was purchased from Air Products Sp, Poland. Methanol for HPLC-super gradient was purchased from POCh (Gliwice, Poland). Acetonitrile, methanol, and water for LC-MS grade were acquired from POCh (Gliwice, Poland). Oxoid (Basingstoke, UK) supplied PDA. All chemicals were of analytical grade. Analytical standards purchased in ready-to-use solutions from Romer Labs (Tulln, Austria) included: ERG, FB_1, FB_2, FB_3, ZEN, DON, 15-AcDON, 3-AcDON, and BEA, which were 100 µg/mL. The β-ZOL concentration was 10 µg/mL. ZEN-14S (100 µg/mL) was purchased in Aokin (Berlin, Germany). Depending on solubility, the standards were dissolved in acetonitrile. All standards were stored in amber glass vials at approximately minus 20 °C. A mixture of all standards necessary for a particular analytical run was prepared immediately before the analysis.

5.2. Plant Material

High-quality dried *L. album* flowers *(Lamii Albi flos)* were purchased from a certified Polish company called Dary Natury located in Podlaskie Voivodeship of Poland (53°4′10.98 latitude and 22°58′2.87 longitude).

5.3. Lamium album Flower Extraction

The extracts were obtained using a supercritical CO_2 extraction method with methanol as a co-solvent (MV-10 ASFE, Waters, Manchester, MA, USA) [26]. The fine-ground samples of *L. album* flowers (9 g) were loaded in the extraction vessel of 25 mL and inserted in the oven set at the desired temperature (50 °C) and pressure (250 bar). The CO_2 flow rate was 4 mL/min, and the methanol flow rate was fixed at 1 mL/min. The extraction time was 180 min, divided into 1st dynamic time of 45 min, a static time of 15 min, and 2nd dynamic time of 120 min. *L. album* extracts were collected in flasks placed in a fraction collection module. The obtained methanolic extracts were transferred into a round bottom flask and evaporated in a vacuum evaporator (Buchi R-215 Rotary Evaporator System, Essen, Germany) to eliminate the methanol; the dried extract was reconstituted with distilled water and stored at −18 °C until it was required for the antifungal assay.

5.4. Description of Fungal Test Pathogens

Isolates of two plant pathogenic *Fusarium* species, *F. proliferatum* PEA 1 and *F. culmorum* KF 846, were used as test pathogens. The strains were initially isolated from pea seeds and wheat kernels, respectively. They were identified by molecular techniques [50], stored in the collection of pathogenic fungi at the Institute of Plant Genetics, Polish Academy of Sciences, Poznan, Poland, and had already been characterized during previous research.

5.5. Determination of the Antifungal Activity of L. album Flower Extracts

The study evaluated the antifungal effects of 2.5, 5, 7.5, and 10% of *L. album* flower extract against *F. culmorum* and *F. proliferatum* growth. For this objective, the poisoned food technique was used [41,51]. This technique involves mixing the prepared *L. album* extract with a cooled PDA (45 °C) medium. Fifteen ml of PDA medium/extract-supplemented medium was poured onto each plate using an automatic dispenser. Using a sterile cork borer, agar discs with mycelia (6 mm in diameter) were cut from the periphery of actively growing regions of the 7-day-old pure cultures and aseptically inoculated at the center of the Petri plates. The controls contained only PDA medium and fungal discs in the middle. All the inoculated plates were incubated at 25 °C in the dark for 10 days, and the radial

mycelial growth was measured after 3, 6, and 10 days. Triplicates were maintained for each concentration and control. The experiment was repeated three times. Finally, the antifungal activity of each extract concentration was calculated in terms of the inhibition percentage of mycelia growth using the following formula reported in previous studies [10,41].

$$\text{The inhibition percentage of fungal growth (\%)} = [(C - L)/C] \times 100 \quad (1)$$

where C is the average diameter of fungal growth on control Petri dishes, and L is the average diameter of fungal growth on Petri dishes with *L. album* flower extracts. Following the incubation period, the contents of each petri dish (PDA with mycelia) were collected and lyophilized. Subsequently, the dried samples were ground into a fine powder and stored at room temperature for subsequent experiments, including the quantification of ERG and mycotoxins.

5.6. Determination of ERG Content

For the ERG quantitation, the procedure described by Uwineza et al. [10] was followed. The dried PDA with mycelium (100 mg) was suspended in 2 mL of methanol in a culture tube and treated with 0.5 mL of 2 M aqueous sodium hydroxide. Samples were irradiated three times in a microwave oven (370 W) for 10 s and then were neutralized with 1 mL of 1 M aqueous hydrochloric acid. Samples were extracted with n-pentane (3 × 4 mL) and transferred to the vials. Extracts were evaporated to dryness in a stream of nitrogen. Before chromatographic analysis, dry residues were dissolved in 1 mL of methanol. The ERG separation was performed on a 3.9 mm Nova Pak C-18, 4 mm column with methanol:acetonitrile (90:10, v/v) as the mobile phase at a 1.0 mL/min flow rate. ERG was detected with a Waters 2996 Photodiode Array Detector (Waters Division of Millipore, Milford, MA, USA) set at 282 nm. The presence of ERG was confirmed by comparing retention times with the external standard and by co-injection of every tenth sample with an ERG standard. The detection limit was 10 ng/g.

5.7. Determination of Mycotoxins by Using UPLC/MS/MS

The mycotoxins were extracted by adding 5 mL of the extraction solvents (acetonitrile:water, 86:16, v/v) to 0.5 g of dried PDA with mycelium; the extraction process involved vortexing for approximately 30 s and mixing with a horizontal shaker for 24 h. Then, the samples were centrifuged at 10,000 rpm for 10 min. After centrifugation, approximately 2 mL of mycotoxin extracts were filtered through a 0.45 µm nylon syringe filter and collected in vials for mycotoxin identification and quantification. For the analysis, the method reported by Uwineza et al. (2022) was followed with some modifications [10]. The analytes were quantitatively analyzed using multiple reaction monitoring, and their identification was confirmed by comparing the retention times and m/z values with those of corresponding standards. The analysis was performed in triplicate.

5.8. Statistical Analysis

The results are presented as the mean (±) standard deviation of three parallel replicates. A one-way analysis of variance was performed to evaluate the significance of differences in the *L. album* flower extracts (2.5, 5, 7.5, and 10%) in the determined ERG and mycotoxin concentrations in PDA mycelium. Subsequently, a post hoc Tukey's honest significant difference (HSD) test with a significance level of $\alpha = 0.01$ was conducted for paired comparisons. Analyses were conducted using the Statgraphics v. 4.1 software package (Graphics Software System, STCC, Inc., Rockville, MD, USA) and GraphPad Prism 9 software.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/toxins15110651/s1, Table S1. The effect of different concentrations of *L. album* flower extracts (2.5, 5, 7.5, and 10%) on *F. proliferatum* mycotoxins after 10 days of incubation at 25 °C on a PDA medium; Table S2. The effect of different concentrations of *L. album*

flower extracts (2.5, 5, 7.5, and 10%) on *F. culmorum* mycotoxins after 10 days of incubation at 25 °C on a PDA medium.

Author Contributions: Conceptualization, P.A.U. and A.W.; Methodology, P.A.U., M.U. and A.W.; Validation, P.A.U., M.U. and A.G.-M.; Formal analysis, P.A.U. and M.U.; Investigation, P.A.U. and M.U.; Resources, Ł.S. and A.W.; Data curation, P.A.U., A.G.-M. and A.W.; Writing—original draft, P.A.U. and A.W.; Writing—review and editing, P.A.U., Ł.S. and A.W.; Visualization, P.A.U. and M.U.; Supervision, A.W.; Project administration, P.A.U. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Polish National Science Centre, grant number 2018/31/B/NZ9/03485.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article and supplementary materials.

Acknowledgments: We would like to express our gratitude to Magdalena Tomaszewska for her support in capturing the photos of Petri dishes with mycelium.

Conflicts of Interest: The authors declare no conflict of interest.

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Statement about the contribution of authors

Publication P4

Uwineza, P. A.; Urbaniak, M.; Stępień, Ł.; Gramza-Michałowska, A.; Waśkiewicz, A.2024. Efficacy of *Lamium Album* as a Natural Fungicide: Impact on Seed Germination, Ergosterol, and Mycotoxins in *Fusarium culmorum* - Infected Wheat Seedlings. *Front. Microbiol.*, 15, 1363204. https://doi.org/10.3389/fmicb.2024.1363204.

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Monika Urbaniak	Methodology, Validation, Formal analysis, Investigation, Visualization	5%	
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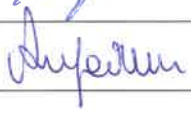
Anna Gramza-Michałowska	Validation, Data curation	5%	
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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 23 February 2024
DOI 10.3389/fmicb.2024.1363204

OPEN ACCESS

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RECEIVED 30 December 2023
ACCEPTED 05 February 2024
PUBLISHED 23 February 2024

CITATION
Uwineza PA, Urbaniak M, Stępień Ł, Gramza-Michałowska A and Waśkiewicz A (2024) Efficacy of *Lamium album* as a natural fungicide: impact on seed germination, ergosterol, and mycotoxins in *Fusarium culmorum*-infected wheat seedlings. *Front. Microbiol.* 15:1363204. doi: 10.3389/fmicb.2024.1363204

Efficacy of *Lamium album* as a natural fungicide: impact on seed germination, ergosterol, and mycotoxins in *Fusarium culmorum*-infected wheat seedlings

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Fusarium culmorum is a major wheat pathogen, and its secondary metabolites (mycotoxins) cause damage to plants, animals, and human health. In the era of sustainable agriculture, eco-friendly methods of prevention and control are constantly needed. The use of plant extracts as biocontrol agents has gained popularity as they are a source of active substances that play a crucial role in fighting against phytopathogens. This study evaluated the impact of *Lamium album* on wheat seed germination and seedling growth. In a pot experiment, the effect of *L. album* on wheat seedlings artificially inoculated with *F. culmorum* was evaluated by measuring seedling growth parameters, and by using chromatographic methods, ergosterol and mycotoxins levels were analyzed. The results showed that the phytotoxic effect of *L. album* flower extracts on wheat seed germination and seedling growth was concentration dependent. The radicle length was also reduced compared to the control; however, *L. album* did not significantly affect the dry weight of the radicle. A slight phytotoxic effect on seed germination was observed, but antifungal effects on artificially infected wheat seedlings were also confirmed with the reduction of ergosterol level and mycotoxins accumulation in the roots and leaves after 21 days of inoculation. *F. culmorum* DNA was identified in the control samples only. Overall, this study is a successful in planta study showing *L. album* flower extract protection of wheat against the pathogen responsible for Fusarium crown and root rot. Further research is essential to study the effects of *L. album* extracts on key regulatory genes for mycotoxin biosynthetic pathways.

KEYWORDS

***Triticum* genus, plant extract, *Lamiaceae*, PCR, trichothecenes, zearalenone**

1 Introduction

Wheat is a crucial cereal crop in the *Triticum* genus, and it serves as a dietary staple for nearly 40% of the world's population (Iqbal et al., 2022). Despite being the second most crucial staple after rice (Singh et al., 2016; Özdemir, 2022), *Fusarium* infections in wheat present a global challenge, causing reduced yield, compromised quality, and the accumulation of harmful mycotoxins (Bota et al., 2021; Riaz Ejaz et al., 2023). *Fusarium culmorum*, a soil-borne fungus (Antalová et al., 2020), is a primary wheat pathogen, with *Fusarium* root rot, *Fusarium* crown rot, and *Fusarium* head

blight (FHB) being particularly threatening due to mycotoxin contamination (Bottalico and Perrone, 2002; Scherm et al., 2013; Antalová et al., 2020). Zearalenone (ZEN) and type B trichothecenes, such as deoxynivalenol (DON), acetyl-deoxynivalenol (3-ADON and 15-ADON), and nivalenol (NIV) (Alisaac et al., 2023) pose severe risks to human health, food security, and economy (Ostry et al., 2017). Therefore, managing *Fusarium* diseases in cereals, especially wheat, is crucial for ensuring food security and safeguarding human health.

Efforts to combat *Fusarium* infections in wheat have involved substantial investments in genetic resistance, crop rotation, and other practices (Özdemir, 2022). However, complete host resistance remains elusive (Wagacha and Muthomi, 2007), underscoring the ongoing importance of fungicides (Zubrod et al., 2019). Synthetic fungicides, while effective, pose risks to human health and the environment (Castro et al., 2020; Seepe et al., 2021), forcing the exploration of natural alternatives, which are vital for sustainable crop cultivation. Plant extracts are recognized as safe and offer a promising solution (Abdallah et al., 2018), containing a blend of compounds, including phenolic acids, flavonoids, tannins, terpenes, and alkaloids, that can collaborate to inhibit the growth of phytopathogenic fungi (Deresa and Diriba, 2023). Moreover, plant extracts are highly effective against a wide range of pests and diseases, are relatively easy and cheap to produce, and show low toxicity against non-target organisms, including humans (Suteu et al., 2020). Significantly, these compounds not only limit fungal growth by inhibiting ergosterol biosynthesis as an indicator of fungal biomass (Perkowski et al., 2008) but also inhibit mycotoxin biosynthesis and trigger plant defense responses (Acheuk et al., 2022). However, to monitor the *in vivo* antifungal properties of a plant extract, it is essential to evaluate the phytotoxic effects on the target plant that requires protection, considering that certain studies have indicated their potential adverse effects.

L. album is a medicinal plant distributed in Europe, Asia, and Africa (Chipeva et al., 2013). To understand the mechanisms of action, researchers identified its chemical constituents and found that it has various chemicals, including iridoid glycosides, phenolic acids, flavonoids, alkaloids, triterpenes, and other compounds with diverse biological properties (Damtoft, 1992; Pereira et al., 2012; Pourmirzaee et al., 2019; Sulborska et al., 2020; Uwineza et al., 2021). In recent *in vitro* research, *L. album* flower extracts demonstrated promising effects against *Fusarium* species, emphasizing its versatile applications (Uwineza et al., 2023). Most studies focus on plant extracts' fungicidal or fungistatic effects (García-Ramírez et al., 2023). However, they often overlook the impact of these extracts on mycotoxin production by pathogenic fungi and phytotoxic effects on the crops. Furthermore, most of these studies are conducted *in vitro* (Kursa et al., 2022; Uwineza et al., 2022), while *in vivo* experiments are necessary to confirm the biocontrol effects of such plant extracts, as the data available is limited. In addition, for the plant extract to be effective, accurate identification of the pathogen is crucial. Identifying pathogens based on observed symptoms can be challenging, especially at the early stages of disease. Therefore, molecular methods, particularly those based on polymerase chain reaction (PCR), offer superior detection capabilities. The technique is a rapid and sensitive tool for detecting and identifying targeted DNA molecules of pathogens in plant tissues (Pszczółkowska et al., 2013).

The study aimed to assess the efficacy of *L. album* flower extract [obtained by using supercritical fluid carbon dioxide ($SC-CO_2$)] on wheat seed germination and analyze its antifungal action against *F. culmorum* in artificially infected wheat seedlings. Our previous research has shown a promising *in vitro* effect of *L. album* against *F. culmorum* and *F. proliferatum* (Uwineza et al., 2023), and the literature has indicated that infections in seedlings and basal stems often originate from seed or soil-borne inoculum, leading to significant yield losses due to damaged seedlings, pre-harvest lodging, and impaired grain filling (Gebremariam et al., 2020). Therefore, controlling *Fusarium* infection from the early stage may promise better harvesting.

2 Results

2.1 Effect of *Lamium album* on wheat seed germination and seedling growth

The effect of *L. album* flower extract on wheat seeds was evaluated after 7 days of incubation. The results showed that *L. album* extracts inhibited wheat seed germination in a concentration-dependent manner (Table 1). The germination percentage was calculated using the Equation 1, where the control sample had a 100% germination percentage, while the treated samples showed a significant difference with a 65% germination percentage for 5% of extracts, while for 10% of extracts, the germination percentage was 45%. Additionally with the Equation 2, *L. album* flower extract reduced the seedling length vigor index from 669.50 to 303.86 compared to the control (2,860.00). It also decreased the seedling weight vigor index from 2.83 to 1.84 compared to the control value of 3.92 obtained using the Equation 3.

Furthermore, the concentration of *L. album* significantly influenced both shoot length ($F = 259.3$, $p < 0.0001$) and root length ($F = 282.5$, $p < 0.0001$) of wheat seedlings, as depicted in Figures 1, 2. However, there was no significant difference in dry matter ($F = 0.7018$, $p = 0.5209$). With an increase in *L. album* concentration, there was a noticeable decrease in shoot and root length.

In specific instances, such as at a 10% *L. album* concentration, the germinated seeds resulted in shoot and root lengths of 4.55 and 2.20 cm, respectively. In contrast, at a 5% *L. album* concentration, the shoot and root lengths were 6.975 and 3.32 cm, respectively, both shorter than the untreated control with shoot and root lengths of 12.88 cm and 15.73 cm, respectively.

TABLE 1 Comparative effects of *L. album* on wheat seed germination.

Sample name	Germination percentage [%]	Seedling length vigor index	Seedling weight vigor index
Control	100[a]	2,860.00[a]	3.92[a]
5% extract	65[b]	669.50[b]	2.83[ab]
10% extract	45[c]	303.86[c]	1.84[b]

Values with different letters are statistically different (values obtained from the mean results of the treatment, $\alpha = 0.05$).

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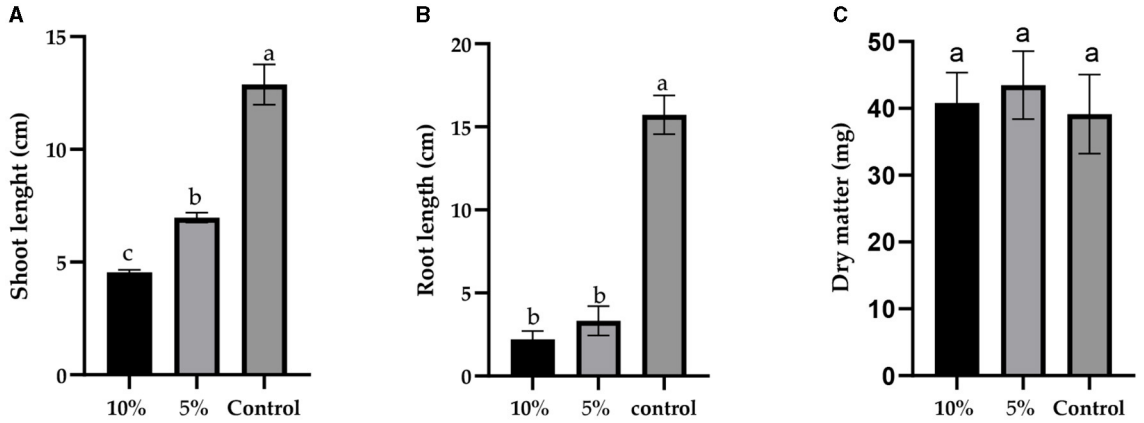

FIGURE 1
The effect of different concentrations of *L. album* extract (5 and 10%) compared to the untreated control: **(A)** the shoot length (cm), **(B)** root length (cm), and **(C)** dry matter (mg) of seedlings after 7 days of growth. Values with different letters are statistically different (mean ± standard deviation, $\alpha = 0.05$).

FIGURE 2
The effects of different concentrations of *L. album* flower extract (5% and 10%) compared to the untreated control (treated with water) on seedlings' shoot and root length after 7 days of incubation.

TABLE 2 **Effect of *L. album* extracts on the growth of infected and non-infected wheat seedlings.**

Sample treatments	Fresh weight [g]	Shoot length [cm]	Root length [cm]	Plant height [cm]	Root weight [g]
Control_ FC	$11.49^{b} \pm 4.47$	$48.50^{a} \pm 2.38$	$48.5^{ab} \pm 6.25$	$97.00^{ab} \pm 7.39$	$3.24^{a} \pm 1.16$
5% _FC	$15.32^{a} \pm 3.83$	$51.25^{a} \pm 2.36$	$45.75^{ab} \pm 8.22$	$97.00^{ab} \pm 8.41$	$2.94^{ab} \pm 0.66$
10% _FC	$11.25^{b} \pm 3.42$	$50.25^{a} \pm 2.06$	$36.25^{b} \pm 6.95$	$86.50^{b} \pm 7.55$	$2.96^{ab} \pm 1.16$
Control_NC	$12.62^{ab} \pm 2.97$	$47.75^{a} \pm 3.86$	$39.25^{b} \pm 6.85$	$87.00^{b} \pm 9.63$	$2.95^{ab} \pm 1.15$
5% NC	$8.53^{b} \pm 1.69$	$49.25^{a} \pm 0.96$	$37.50^{b} \pm 8.74$	$86.75^{b} \pm 8.96$	$1.04^{b} \pm 0.48$
10% NC	$8.15^{b} \pm 2.43$	$48.00^{a} \pm 2.16$	$56.25^{a} \pm 16.78$	$104.25^{a} \pm 15.39$	$1.32^{b} \pm 0.13$

All the values are the mean of four replicate ± standard deviation. Values with different letters are statistically different ($\alpha = 0.05$); **Control_FC** - seedlings inoculated with *F. culmorum* without extract, **5% _FC and 10% _FC** - seedlings treated with extracts and inoculated with *F. culmorum*, **Control_NC** - seedlings treated with water without the inoculation of *F. culmorum*, and **5% NC and 10% NC** - seedlings treated with extract without the inoculation of *F. culmorum*.

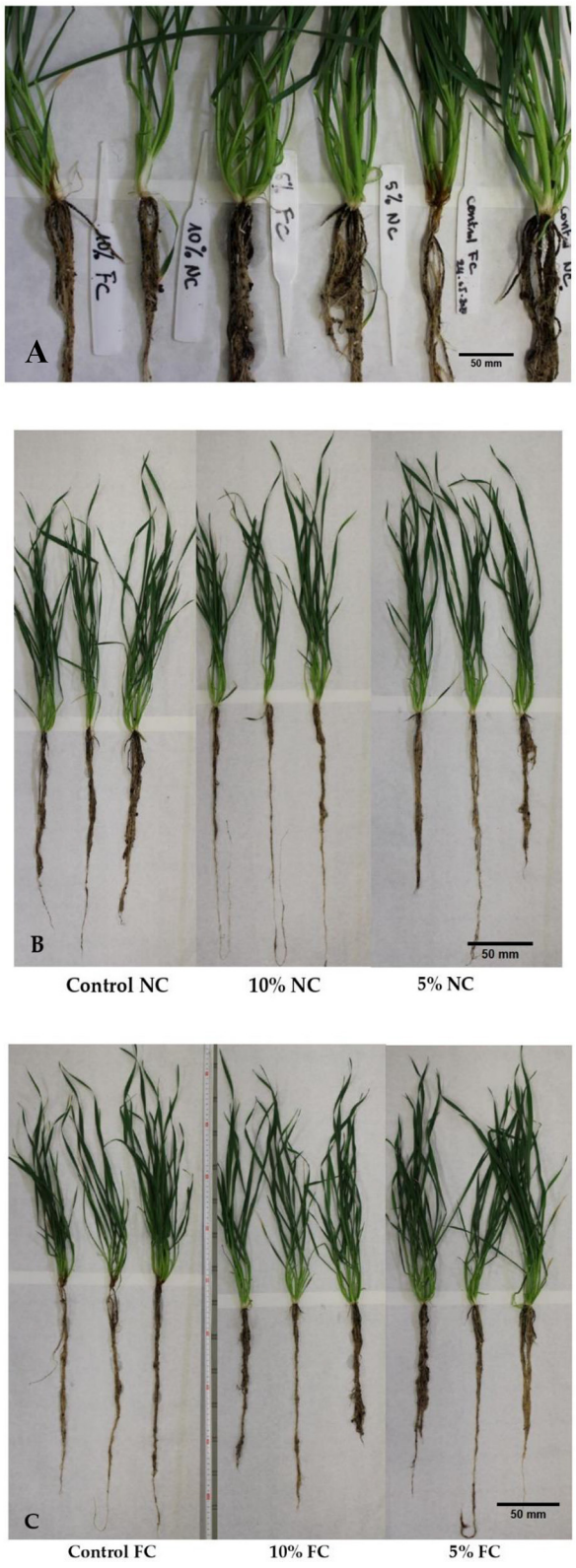

FIGURE 3
Differences in root phenotype among the harvested wheat seedlings after 21 days of infection: **(A)** comparative observation of the infected and non-infected seedlings, **(B)** non-infected seedlings, and **(C)** infected seedlings.

2.2 Comparative effects of *L. album* extract on the physiological growth parameters of wheat seedlings in the presence and absence of *Fusarium culmorum* infection

Physiological growth parameters of wheat seedlings were variably influenced by *L. album* flower extract and *F. culmorum* infection. With the data collected after 21 days of artificial infection of wheat seedlings, there are slight differences between the seedling root weight of the pathogen-inoculated control (Control_FC) and the inoculated and treated with 5% and 10% of *L. album* extract (5% _FC and 10% _FC) with a mass drop of approximately (−0.3 g). However, root weight loss was observed in non-infected seedlings treated with *L. album* extract (5% and 10% NC), with a mass drop of (−1.8 g) compared to the control. Similar effects were also observed in root length, except for non-infected seedlings treated with 10% of extracts (10% NC), which showed the longest roots (Table 2, Figure 3). The samples treated with 10% extract showed a stunting effect of *F. culmorum* on seedling root development, where the infected seedlings (10% _FC) showed a decrease in root length compared to non-infected seedlings (10% NC) but increased root weight. This reduction may signify a positive impact of *L. album* flower extract in combating *Fusarium*. Shorter roots could be a defense response, limiting *Fusarium* invasion and strengthening plant resistance. This observation underscores the potential antifungal efficacy of *L. album* extract.

In summary, *L. album* flower extract and *F. culmorum* infection affected the whole plant. Five percent extract yielded 15.32 g of fresh weight of the seedling compared to 11.25 g for 10% extract. Furthermore, total plant height was also affected, and 5% of samples showed greater height than the 10% samples. In addition, visual differences between the seedlings were observed, with the infected seedlings displaying infection symptoms and non-infected wheat seedlings showing no symptoms (Figure 4).

2.3 *Fusarium* spp. identification

Molecular identification of all fungal strains that might be present in the collected seedlings was performed using sequence analysis of the PCR-amplified fragments PCR. The DNA regions were amplified with EF728M and Tef1α primers and were subsequently sequenced. Amplified DNA fragments were compared with reference genes from the GenBank Database to confirm species identification. The results showed that the isolated strains in the contaminated wheat seedlings were *Fusarium* spp., as expected. However, isolates in the roots of the control group were *F. culmorum* (Strains -FC1Croot, and FC-C2root with 96.4% and 96.81% identical bases, respectively). For roots of the seedlings treated with *L. album* extracts, the identified fungi were *F. oxysporum* (Strains - FC5.4.1R and FC10.5.1G with 97.86% and 98.2% identical bases, respectively). In addition, no significant similarities in fungal species were found in infected seedling leaves as well as in non-infected seedling leaves and roots (data not shown).

FIGURE 4
F. culmorum symptoms in the treated seedlings and the control group after 21 days of infection.

TABLE 3 Comparative effects of *L. album* on the ergosterol content [μg/g] and its reduction [%] between the infected and non-infected wheat seedlings.

Treatments*	Ergosterol content [μg/g]		
	Control	5% extract	10% extract
Leaves_NI	$9.89^{d} \pm 1.62$	$8.81^{cd} \pm 1.04$ (10.94%)	$7.72^{cd} \pm 2.44$ (21.92%)
Roots_NI	$8.58^{cd} \pm 0.39$	$5.51^{cde} \pm 1.00$ (35.80%)	$4.85^{de} \pm 0.80$ (43.51%)
Leaves_I	$25.11^{b} \pm 4.17$	$5.19^{cde} \pm 1.54$ (79.31%)	$3.87^{e} \pm 1.83$ (84.57%)
Roots_I	$41.46^{a} \pm 18.19$	$19.85^{bc} \pm 7.34$ (52.14%)	$14.30^{bcd} \pm 2.85$ (65.52%)

All the values are the mean of four replicate ± standard deviation. Values with different letters are statistically different ($\alpha = 0.05$); *__Leaves_NI:__ leaves of non-infected seedlings; **Roots_NI**: roots of non-infected seedling; **Leaves_I**: leaves of the infected seedlings; **Roots_I**: roots of the infected seedlings.

TABLE 4 Effects of *L. album* on the mycotoxin biosynthesis [ng/g] on the infected wheat seedlings.

Mycotoxins	Treatments	Seedling parts	
		Leaves_I	Roots_I
DON	Control	nd^{b}	$18.90^{a} \pm 13.37$
	5%	nd^{b}	nd^{b}
	10%	nd^{b}	nd^{b}
3- and 15-AcDON	Control	nd^{b}	$30.31^{a} \pm 12.10$
	5%	nd^{b}	nd^{b}
	10%	nd^{b}	nd^{b}
ZEN	Control	$31.30^{b} \pm 6.70$	$56.01^{a} \pm 8.48$
	5%	nd^{d}	$10.00^{c} \pm 2.48$
	10%	nd^{d}	$7.28^{c} \pm 1.73$
ZEN-14S	Control	$46.26^{a} \pm 4.66$	$51.68^{a} \pm 12.33$
	5%	nd^{c}	$12.90^{b} \pm 3.59$
	10%	nd^{c}	$10.87^{b} \pm 1.05$

All the values are the mean of four replicates ± standard deviation. Values with different letters are statistically different ($\alpha = 0.05$). *__Leaves_I:__ leaves of the infected seedlings; **Roots_I**: roots of the infected seedlings. DON, deoxynivalenol; 3- and 15-AcDON, 3- and 15-acetyl deoxynivalenol; ZEN, zearalenone; ZEN-14S, zearalenone-14-sulfate; nd, not detected.

2.4 Effect of *L. album* flower extracts on *Fusarium* growth

The influence of extracts on the growth and development of *Fusarium* was measured by ergosterol (ERG) content, which is an indicator of fungal biomass. The HPLC/PDA analysis successfully detected the presence of ergosterol in both the roots and leaves of the harvested wheat seedlings (Table 3). ERG was present both in non-infected and infected seedlings. In the non-infected seedlings, the control without the extract showed the highest ERG level in leaves and roots, the ones with 5% of the extract being lower and the lowest for 10%, meaning that endophytic fungi were present in the plant tissue. In both cases (roots and leaves), the extract inhibited fungal development inside the plant. ERG levels in those seedlings were significantly lower compared to the infected seedlings. Similarly, in the case of artificially infected seedlings, the control without the extract had the highest level of ergosterol in roots and leaves. However by using the Equation 4, a significant ERG reduction was observed in the samples treated with 10% extract, with the highest reduction of up to 84.57% in the leaves and 65.52% in the roots. For the 5% extract, ergosterol was diminished by 52% in the roots and 79.31% in the leaves. This showed that the application of *L. album* extracts led to a notable lowering of ERG levels in the infected seedlings. Statistically, significant differences were observed between the 5% and 10% of extract, as determined by ANOVA with *post-hoc* Duncan test at $p < 0.05$.

2.5 Effects of *L. album* flower extracts on mycotoxins biosynthesis in wheat seedlings artificially infected with *F. culmorum*

Mycotoxin biosynthesis is one of the challenging phenomena associated with fungal infection. Our study analyzed the effects of *L. album* flower extracts on the mycotoxins content in the roots

and leaves of seedlings artificially infected with *F. culmorum* as well as in non-infected seedlings using the LC-MS/MS technique. Four commonly synthesized mycotoxins of *F. culmorum*, namely DON, 3- and 15-AcDON, ZEN, and ZEN-14S were quantified (Table 4). Notably, no mycotoxins were identified in the non-infected wheat seedlings.

A reduction of mycotoxin accumulation was noted in the seedlings treated with *L. album* flower extracts. In the roots, there were significantly higher amounts of all mycotoxins compared to the leaves, with lower production of DON and 3- and 15-AcDON than ZEN and ZEN-14S. *L. album* caused complete inhibition (100%) of DON and 3- and 15-AcDON in the roots. While ZEN was entirely inhibited in the leaves, in the roots decreased within the range of 10.00 to 7.28 ng/g compared to the control value of 56.01 ng/g, representing a reduction of 82.14% for the 5% and 87.41% for the 10% of *L. album* extract. Regarding ZEN-14S, complete inhibition occurred in the leaves, but in the roots, its concentration decreased within the range of 12.90 to 10.87 ng/g compared to the control value of 51.68 ng/g. This reduction was equivalent to 75.03% for 5% concentration and 78.97% for 10%. Overall, a significant impact of *L. album* on mycotoxin biosynthesis was shown, resulting in a noteworthy reduction in mycotoxin concentrations compared to the control, regardless of the extract concentration (differences were insignificant at $p < 0.05$ for all produced mycotoxins).

2.6 Correlation between ergosterol and the produced mycotoxins (Pearson r correlation)

Pearson correlation (Pearson's r) assessed the linear relationship between ergosterol content and mycotoxins in the infected wheat seedlings. The analysis showed that ergosterol content positively correlated with the synthesized mycotoxins, and it was statistically significant at $p < 0.05$. Furthermore, highly positive correlations were found between the concentrations of DON and 3- and 15-AcDON and between ZEN and ZEN-14S (Figure 5). Compared to this, weaker positive correlations were observed between DON and ZEN-14S ($r = 0.57$).

3 Discussion

The germination capability of wheat seeds serves as a crucial indicator reflecting their quality, viability, and overall potential (Hassani et al., 2019). In our study, *L. album* decreased seed germination and seedling growth after seven days of incubation compared to the control. This effect was in agreement with other studies, showing reduced wheat germination of seeds treated with *Ulva linza* or *Corallina officinalis* seaweeds (Hamouda et al., 2022), *Flaveria bidentis* (Dai et al., 2022) and aqueous extracts of weed plants: *Hyptis sauveolens* (L.), *Ricinus communis* (L.), *Alternanthera sessilis* (L.), *Ipomoea carnea* (Jacq), *Malachra capitata* (L.), and *Cymbopogon citrutus* (Stapf) (Joshi and Joshi, 2016). The decrease in germination percentage could be attributed to the potential phytotoxic or allelopathic effects of the extracts (Joshi and Joshi, 2016; Ma et al., 2011; Dai et al., 2022; Hamouda et al., 2022; Rys et al., 2022), such as the harmful or inhibitory impact that plant extracts may have on plant tissues or biological processes (Ma et al., 2011; Werrie et al., 2020) or chemical interactions between plants, which may be direct or indirect, beneficial or detrimental (Joshi and Joshi, 2016; Aurelio et al., 2022). The control group exhibited the longest wheat seedlings, with the lowest recorded dry weight compared to the treated wheat seeds. Although plant extracts mildly inhibited seedling weight loss (Figure 1), the effect was not significantly different from the control at p

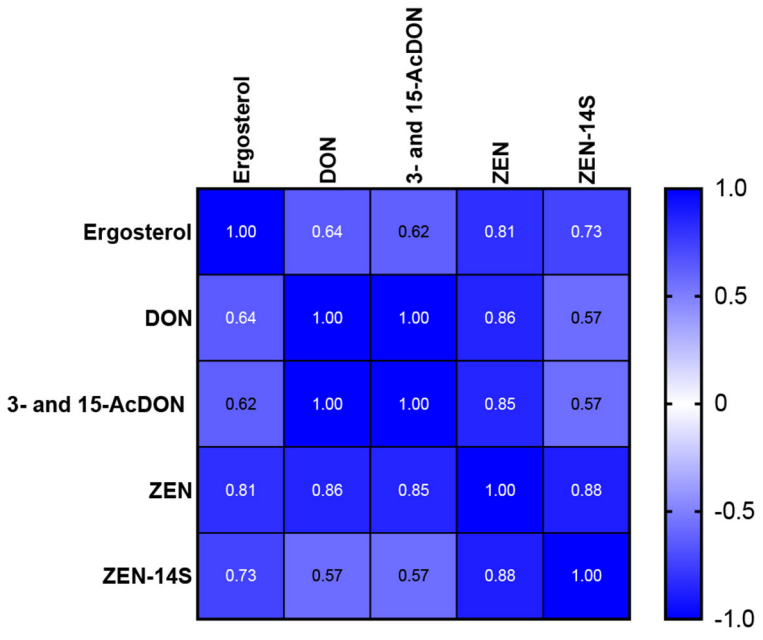

FIGURE 5
Pearson correlation between ergosterol content and biosynthesized mycotoxins in the infected wheat seedlings.

< 0.05. The observed reduction in plant growth under natural conditions may be attributed to seed competition for water, minerals, oxygen, or root system space (Rys et al., 2022). Some plant extracts, despite their beneficial properties, can also contain compounds that hinder seed germination or seedling growth (Bota et al., 2021). Hamouda et al. (2022) investigated the effect of wheat seed priming with the aqueous extracts of *Ulva linza* and *Corallina officinalis* seaweeds; higher concentrations inhibited seedling growth and caused specific chromosomal abnormalities, while low concentrations of seaweed extract as a priming treatment improved wheat seedling growth, physiological features and had a greater capacity to boost bioavailable macro- and micronutrients to the plant (Hamouda et al., 2022).

F. culmorum can impact plants across different developmental stages, leading to seedling blight and root rot, hindering seedling emergence and overall plant development (Pastuszak et al., 2021). This pathogenic fungus infects wheat plants, leading to foot rot symptoms, particularly affecting the roots and lower stems. The severity of the disease varies, with early infections causing pre- and post-emergence seedling death, while later infections result in brown lesions on the lower stems and tiller abortion (Scherm et al., 2013). While FHB is often a primary focus in research due to its economic impact and mycotoxin production (Bottalico and Perrone, 2002; Scherm et al., 2013; Antalová et al., 2020), foot rot caused by *F. culmorum* is a significant issue that requires attention (Scherm et al., 2013). The presence of *L. album* and *F. culmorum* infection caused stress to the growing seedlings and by responding to these biotic stresses, seedlings produced many short roots that have influenced the weight and length of the roots (Table 2). Roots are crucial for nutrient uptake and overall plant health. The observed decrease in root length for the seedling treated with 10%_FC may be a defense response triggered by the plant in the presence of *Fusarium*. *L. album* extract might influence this response positively, aiding the wheat seedling in resisting fungi infection. Moreover, shorter roots may limit the pathogen's ability to invade the plant's vascular system, acting as a physical barrier. While a decrease in root length might be perceived as an adverse effect of *Fusarium* infection in wheat, as noted in a study conducted by Saad et al. (2023), it could be a strategic adaptation to strengthen the plants' defense mechanisms induced by plant extract against *Fusarium* spp, ultimately contributing to enhanced resistance. Previous research showed that seedlings exhibiting moderate vigor were more susceptible to infection than those with high vigor and exhibited a significant height increase when subjected to inoculation with *F. graminearum* spores (Zhou et al., 2018). Similarly, the application of essential oil disturbed the growth of seedlings' roots and reduced the vigor index (Grzanka et al., 2021). Seedlings protected with *L. album* extracts showed no symptoms. In contrast, the control samples exhibited visible symptoms, such as root browning, yellowing of the first leaves, and visible mycelia on the above-ground part of the seedlings (Figure 4). Previous studies reported that the early infection reduced wheat seedling growth as *F. culmorum* can effectively penetrate seedling roots, migrate from hypocotyl to the upper stem internodes and leaves, colonize the host's tissue and cells, block the vascular bundles, disturb nutritional supply and metabolic processes (Kthiri et al., 2021). In another study, three durum wheat genotypes at seedling and full anthesis stage *F. culmorum* infection significantly decreased chlorophyll a, b, and carotenoid contents in the leaves (Pastuszak et al., 2021). *L. album* may also cause adverse effects against *F. culmorum*, inducing toxin accumulation, especially under conditions unfavorable for fungal growth or at low extract concentrations (Tretiakova et al., 2022).

Ergosterol, being integral to fungal cell membranes, serves as an indicator of fungal biomass and is vital for maintaining cell function and integrity (Perkowski et al., 2008; Uwineza et al., 2022). Severe ERG depletion may influence cell growth and proliferation (Abhishek et al., 2015). Here, the effect of *L. album* extract was evident, as shown by the ERG reduction in infected seedlings. Low ERG levels in non-infected seedlings could be attributed to endophytic fungi—microorganisms living within plant tissues without visible disease symptoms (Abaya et al., 2021; Noel et al., 2022). PCR-based identification confirmed *F. oxysporum* in the root of infected samples treated with *L. album* flower extracts. Also, no mycotoxins were identified in those samples. Noel et al. (2022) reported *F. oxysporum* as one of the fungal endophytes that significantly increased seed weight and lowered the accumulation of DON compared to *F. graminearum*-infected wheat heads without endophyte (Noel et al., 2022). *L. album* extract suppressed the growth of pathogenic and endophytic fungi in a concentration-dependent manner. This inhibitory effect could be attributed to the active compounds in *L. album* flower extracts (Pereira et al., 2012; Pourmirzaee et al., 2019; Sulborska et al., 2020; Uwineza et al., 2021). Detrimental impact on ERG content confirmed a significant disruption or biosynthesis blockage within the plasma membrane of *F. culmorum*. This finding is in agreement with some of the previous studies that have reported a decrease in ERG content in infected samples after the application of plant extracts (Abhishek et al., 2015; Uwineza et al., 2022). Leaf extract of *Solanum torvum* Swartz. decreased ERG production by the *Fusarium verticillioides* strain (Abhishek et al., 2015). Previous *in vitro* study also showed *L. album* flower extract activity against *F. culmorum* and *F. proliferatum,* reducing ERG content (Uwineza et al., 2023).

Mycotoxigenic fungi can simultaneously produce various compounds (Streit et al., 2012). To monitor whether *L. album* extract alters the mycotoxin biosynthesis in wheat seedlings, an analysis was conducted, revealing that the content of mycotoxins, including DON, 3- and 15-AcDON, ZEN, and ZEN-14S, commonly associated with *F. culmorum* contamination (Bryła et al., 2018; Uwineza et al., 2022), were reduced both in roots and leaves of seedlings treated with *L. album* extract. Abbas and Yli-mattila (2022) showed that the methanolic extract of the medicinal plant *Zanthoxylum bungeanum* successfully decreased *F. graminearum* growth and abrogated DON production in wheat heads (Abbas and Yli-mattila, 2022). Similarly, Shcherbakova et al. (2018) found no increase in DON, AcDON, and ZEN content when *F. culmorum* and *F. graminearum* developed on extract-treated wheat seeds and seedlings (Shcherbakova et al., 2018).

DON and its acetylated forms 3- and 15-AcDON were completely inhibited by *L. album*. Correlations between DON and its acetylated forms were highly significant, which is in accordance with the previous research (Sunic et al., 2021), where DON highly

correlated with 3-AcDON in winter wheat, and its production by *F. culmorum* is believed to play a role in pathogenesis (Morimura et al., 2020). Mycotoxin accumulation affects germination rates, seedling growth, pathogen aggressiveness, and overall disease severity (Bruins et al., 1993; Scherm et al., 2011; Winter et al., 2019). Scherm et al. (2011) showed that DON is an aggressiveness factor in *F. culmorum* stem base infections of durum wheat, whereby disease incidence was decreased by 40%−80% when the trichothecene regulatory gene *Tri6* was silenced (Scherm et al., 2011). Similarly, Winter et al. (2019) showed that high DON- and 3-AcDON-producing isolate led to more severe symptoms and 20 times more significant colonization of the stem base than isolates that produced less DON/3-AcDON (Winter et al., 2019). A 10-fold higher accumulation of ZEN and ZEN-14S in infected seedling roots was observed compared to DON, which is consistent with previous findings (Ksieniewicz-Wozniak et al., 2021). It can be explained by *L. album* extract ability to regulate the enzymes involved in the synthesis or modification of ZEN and its derivatives compared to the ones that process DON. Additionally, the accumulation of ZEN and ZEN-14S over DON may be favored, according to a study that investigated the effect of compactin on mycotoxin production and the expression of associated biosynthetic and regulatory genes in toxigenic *F. culmorum*. Their findings showed that compactin had a suppressing effect on DON and ZEN differently due to its effect on various genes involved in the biosynthesis of these mycotoxins (Stakheev et al., 2022). These intricate interactions could lead to differential expression of genes related to mycotoxin biosynthesis and modification, resulting in the observed variations in mycotoxin levels (Scherm et al., 2011). However, even though ZEN and ZEN-14S were highly synthesized, the application of *L. album* significantly reduced them compared to the control. ZEN was reduced to 82.14% for 5% and 87.41% for 10% of *L. album* extract, and ZEN-14S was decreased to 75.03% for 5% and 78.97% for 10%, confirming the mechanism of action of *L. album* extract on the reduction of mycotoxin biosynthesis (Jafarzadeh et al., 2022). A strong correlation between ZEN and ZEN-14S was observed, while a weaker correlation occurred among DON, ZEN, and ZEN-14S, which is in accordance with previous research (Birr et al., 2021). ZEN can be modified (e.g., via conjugation) in plants, fungi, and animals through phase I and phase II metabolism (Veršilovskis et al., 2019). This conjugated form (ZEN-14S) is approximately 60 times more estrogenic than ZEN and can be easily hydrolyzed to ZEN in the gastrointestinal tract, thereby increasing exposure to ZEN (Veršilovskis et al., 2019). A high ZEN-14S/ZEN ratio in the malted wheat suggests a possibility of *Fusarium* converting ZEN into a phase II metabolite through sulfation reactions (Ksieniewicz-Wozniak et al., 2021). Wheat infections occur during or shortly after flowering (Wegulo, 2012); however, our study revealed that mycotoxin accumulation can start at the initial stages of infection.

In this study, we have presented the dual effect of *L. album* extracts, which acted as antifungal on *F. culmorum* in wheat seedlings and showed the phytotoxic effect on seed germination. Composition and concentration of *L. album* extracts can be essential here, as they have not been analyzed until now, but similar results have been reported previously (Bayar and Yilar, 2019; El-Alam et al., 2020; Mehdizadeh et al., 2020). El-Alam et al. (2020) reported that all tested essential oils (EO) presented antifungal properties against *F. culmorum* by direct contact and volatility assays. However, all tested EO have also shown a phytotoxic activity, either by inhibiting seed germination or affecting root elongation of rye and lettuce (El-Alam et al., 2020). Therefore, carefully considering and optimizing the extract concentration and application method is advised to ensure that the antifungal benefits outweigh any potential phytotoxic effects of the plant extracts.

4 Conclusions

The present study highlights the dual impact of *L. album* flower extracts on wheat seed germination, seedling growth, and *Fusarium* infection control. While exhibiting phytotoxic and allelopathic effects that hindered seed germination and seedling growth, the extracts effectively controlled *F. culmorum* infection and reduced visible symptoms. The anti-mycotoxigenic potential of *L. album* flower extracts was evident in the complete inhibition of DON, 3- and 15-AcDON, and the substantial reduction of ZEN and ZEN-14S in artificially infected wheat seedlings. The reduction of ergosterol content in the seedling roots confirmed the disruptive impact on *F. culmorum*, emphasizing the potential of *L. album* as a biocontrol agent against *Fusarium* mycotoxins. The study highlights intricate molecular mechanisms at play and emphasizes the need for further exploration of the effect of *L. album* extracts on key regulatory genes for mycotoxin biosynthesis. Despite the observed phytotoxicity, the study suggests the potential of *L. album* extracts as a biocontrol agent, stressing the importance of optimizing concentration and application methods in agricultural practices. Overall, this research contributes valuable insights into the sustainable use of *L. album* in combating *Fusarium* species in wheat cultivation, with further exploration required for effective implementation.

5 Materials and methods

5.1 Plant extraction

The material used for the extraction was dried *Lamium album* flowers purchased from a certified Polish company called Dary Natury located in Podlaskie Voivodeship of Poland (53°4′10.98 latitude and 22°58′2.87 longitude). The extraction method used was the supercritical fluid extraction technique using methanol as a co-solvent, as described in the previous study (Uwineza et al., 2022). Subsequently, the obtained extracts were evaporated in a vacuum evaporator (Buchi R-215 Rotary Evaporator System, Germany) at 40°C to eliminate the methanol; the dried extract was reconstituted in a measured volume of distilled water and stored at −18°C until antifungal assays.

5.2 Studied material

Healthy wheat grains (*Triticum aestivum* L. cultivar Arkadia) were provided by the Plant-Pathogen Interaction Team, Institute of Plant Genetics, Polish Academy of Sciences, Poznan, Poland.

Fungal isolate of *F. culmorum* KF 846 was obtained from the collection of the Plant-Pathogen Interaction Team, Institute of Plant Genetics, Polish Academy of Sciences, Poznan, Poland. The tested strain was cultured in 9 cm Petri dishes on potato dextrose agar medium (PDA, BioShop, Burlington, ON, Canada) at 28°C for 7 days.

5.3 Standards, chemicals and reagents

Carbon dioxide (CO_2, SFE grade), contained in a dip tube cylinder, was purchased from Air Products Sp, Poland. Methanol for HPLC-super gradient was purchased from POCh (Gliwice, Poland). Acetonitrile, methanol, and water for LC-MS grade were acquired from POCh (Gliwice, Poland). Dream Taq green PCR master mix kit was purchased from Thermo Scientific (Vilnius, Lithuania). Analytical standards including ERG, ZEN, DON, 15-AcDON, and 3-AcDON were purchased in ready-to-use solutions from Romer Labs (Tulln, Austria), and ZEN-14S (100 µg/mL) purchased in Aokin (Berlin, Germany). Depending on solubility, the standards were dissolved in acetonitrile. All standards were stored in amber glass vials at approximately −20°C. A mixture of all standards necessary for a particular analytical run was prepared immediately before the analysis.

5.4 Effect of *Lamium album* flower extract on seed germination and seedling growth

The effect of *Lamium album* flower extract on the germination of wheat kernels was determined according to Al-Khafajy et al. (2022) with some modifications. Ten mL solution of *L. album* flower extract was prepared at 5% and 10% concentrations, then added to sterilized glass plates with tissue paper containing sterile 10 grains/plate; the control samples were prepared with 10 mL of distilled water, and aseptically prepared plates (four plates for each treatment) were incubated under controlled whirlpool chamber at 28°C for 7 days. Germination percentage was calculated using the following formula (Equation 1):

$$\text{Germination percentage (\%)} = (\text{number of healthy seedlings/total number of the seed}) \times 100 \quad (1)$$

The root and shoot length of the seedlings were measured after seven days of germination. Then, all seedlings were collected and freeze-dried to determine the seedling dry weight (g). Also, seedling length vigor index (SLVI) and seedling weight vigor index (SWVI) relations were determined by the following Equations 2, 3 (Hassani et al., 2019):

$$\text{SLVI} = (\text{mean shoot length (cm)} + \text{mean root length (cm)}) \times \text{percentage of seed germination} \quad (2)$$

$$\text{SWVI} = \text{dry weight seedling (g)} \times \text{percentage of seed germination} \quad (3)$$

5.5 Effect of *Lamium album* flower extracts on wheat seedlings against *F. culmorum* in controlled conditions

Ten wheat grains were surface sterilized using a 0.6% (v/v) bleach (sodium hypochlorite) solution, followed by rinsing with distilled water three to four times. Subsequently, 10 mL of *Lamium album* flower extract was prepared at 5% and 10% concentrations, then added to sterilized glass plates with tissue paper containing 10 sterile grains/plate. The control samples were prepared with 10 mL of distilled water. Aseptically prepared plates (four plates for each treatment) were incubated under a controlled whirlpool chamber at 28°C for 7 days to germinate. Plastic pots (13 cm in height) were prepared and filled with a sterile potting mix of soil, sand, and horticulture vermiculite fine (60%, 30%, and 10%; v/v/v) each. Each pot was then planted with a germinated grain (7-day-old seedling) treated with water, and grains treated with *L. album* extracts at 5 and 10% were marked accordingly. The pots were divided into two groups (four pots for each treatment): the first group served as the control, while in the second group, each pot was inoculated with 100 ml of the prepared spore suspension of *F. culmorum* containing 10^6 conidial spores/mL after 10 days of potting. All the pots were arranged on the control room benches, maintained under a natural photoperiod (16 h of light/8 h of darkness) at 23 ± 4°C and with 45% humidity. The seedlings were irrigated with sterile water (100 ml) after 2 days. Daily observations were made to detect any disease symptoms, and the experiment was concluded on the 21st day after inoculation. Both underground (roots) and aerial parts (leaves) of the seedlings were measured (length and weight), collected separately, and stored for further analysis. The experiment comprised four replicates for each treatment, including infected and non-infected wheat seedlings treated with *L. album* flower extracts at 0% control, as well as 5% and 10% concentrations. This process was repeated for reproducibility, and the results represent the mean of two distinct experiments.

5.5.1 DNA extraction, molecular identification, PCR primers, and DNA sequencing

5.5.1.1 Isolation and purification of fusarium species

To isolate *Fusarium* spp. from the collected seedling parts (roots and leaves) from each treatment, a 0.6% (v/v) bleach (sodium hypochlorite) solution was used for surface sterilization, followed by rinsing with distilled water three to four times. Subsequently, the sterilized samples were placed on sterile plates with PDA and incubated at 28°C in the dark for 7 days. The resulting colonies were transferred until purified isolates were obtained. All fungi were maintained on PDA medium at 4°C and sub-cultured monthly until DNA analysis.

5.5.1.2 DNA extraction and *Fusarium* sp. identification

Genomic DNA was extracted using a modified method with the hexadecyltrimethylammonium bromide (CTAB) (Urbaniak et al., 2020). Mycelium scraped from a 7-day-old PDA culture was ground to a fine powder with liquid nitrogen, and 800 µl

of CTAB solution was added to each Eppendorf tube, 150 μl of chloroform-isoamyl alcohol (24:1), then mixed gently. Then, 4 μl of β-mercaptoethanol was added, and samples were incubated in the water bath for 20 min at 65°C. After cooling for 5 min, 150 μl of chloroform-isoamyl alcohol (24:1) was added and mixed gently. The samples were centrifuged for 20 min at 12,000 rpm and 4°C. The supernatant was supplied with 60 μl of 3M sodium acetate and 1,000 μl of ethyl alcohol and precipitated 20 min at −23°C. Then, the samples were centrifuged for 20 min at 12,000 rpm, air dried for 1 h, and re-dissolved in 150 μl of TE buffer pH 8.

Polymerase chain reactions (PCRs) were carried out using Dream Taq green PCR master mix with the help of a C-1000 Touch thermal cycler (Bio-Rad, Hercules, CA, USA). The conditions for PCR amplification were described earlier (Urbaniak et al., 2020), where primers EF728M (5′-CATCGAGAAGTTCGAGAAGG-3′) and Tef1α (5′-GCCATCCTTGGAGATACCAGC-3′) were used. Each reaction tube contained 25 μl reaction mixture made of 12.5 μl of Dream Taq green PCR master mix (Thermo Scientific, Vilnius, Lithuania), 11 μl of water, nuclease-free (Thermo Fisher Scientific, Vilnius, Lithuania), 0.1μl of each primer pairs (EF728M and Tef1α; Thermo Fisher Scientific, Vilnius, Lithuania), and 1.5μl of DNA extract samples. The amplification conditions were as follows: initial denaturation of 3 min at 95°C, 35 cycles of (30 s at 95°C, 30 s at 56°C), and 1 min at 72°C with a final elongation of 15 min at 72°C. Amplification products were electrophoresed in 1.5% agarose gels (EURx Ltd., Gdansk, Poland) in 1×TBE buffer (0.178 mol L-1 Tris-borate, 0.178 mol L-1 boric acid, 0.004 mol L-1 EDTA from Sigma-Aldrich, Steinheim, Germany) containing 5 μl of ethidium bromide. Fragments were visualized under a UV transilluminator and photographed using a PolyDoc System. GeneRuler TM 100bp DNA Ladder Plus was used to establish the molecular weight of the products.

For sequence analysis, PCR-amplified DNA fragments were purified with exonuclease I (Vilnius, Lithuania) and Fast AP thermosensitive alkaline phosphatase (Vilnius, Lithuania) using the following program: 15 min at 37°C and 15 min at 85°C. The strand was labeled using a BigDye^{Tm} Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Vilnius, Lithuania), according to the previously described protocol (Stepień and Waśkiewicz, 2013) and precipitated with 70% ethanol. Sequence reading was performed using Applied Biosystems equipment. Sequences were edited using Chromas v. 1.43 (Technelysium, Tewantin, Australia) and analyzed using the BLASTn algorithm. Sequences were deposited in GenBank and will be publicly available.

5.5.2 Chemical analysis

5.5.2.1 The ERG content analysis

After collecting the seedling's roots and leaves, the materials were lyophilized and ground into fine powder. The concentration of ERG in the samples was determined by comparing the retention time of the analyte with that of an external standard (Waśkiewicz et al., 2014). The method had a detection limit of 10 ng/g. The ergosterol reduction percentage was calculated using the following formula (Equation 4):

$$\text{ERG reduction (\%)} = [(\text{control-treatment})/\text{control}] \times 100 \quad (4)$$

5.5.2.2 Mycotoxins analysis

Mycotoxin extraction was performed by adding 5 mL of the extraction solvents (acetonitrile: water, 86:16, v/v) to 0.5 g of dried roots or leaves of the infected and non-infected wheat seedlings, vortexing (for about 30 s) and mixing using a horizontal shaker for 24 h. After extraction, the samples were centrifuged at 7,500 rpm for 10 min. Then, approximately 2 mL of supernatant was filtered through a 0.2 μm syringe filter (Chromafil, Macherey-Nagel, Duren, Germany) and poured into vials for chromatographic analysis. For the analysis, the method reported by Uwineza et al. (2022) was followed with some modifications (Perczak et al., 2020). The compounds were quantitatively analyzed using multiple reaction monitoring. The mycotoxin concentrations (μg/g) were calculated using a calibration curve based on commercial single-component preparations of DON, 3- and 15-AcDON, ZEN, and ZEN-14S. All samples were analyzed in triplicate.

5.5.3 Statistical analysis

The experimental design consisted of four replicates of each treatment, and each experiment was repeated twice. The results were interpreted as mean standard deviation. Analysis of variance (ANOVA-One way) was applied for the statistical analysis of experimental data using the Statgraphics v. 4.1 software package (Graphics Software System, STCC, Inc., Rockville, MD, USA), and each experimental value was compared with the corresponding control. Where there was statistical significance ($p < 0.05$), the mean values were further separated using Duncan's multiple-range test. GraphPad Prism9 was used for the graphs.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

PAU: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. MU: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. ŁS: Resources, Writing – review & editing. AG-M: Data curation, Validation, Writing – review & editing. AW: Conceptualization, Data curation, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This

research was funded by the Polish National Science Centre, grant number 2018/31/B/ NZ9/03485.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Statement about the contribution of authors

Publication P5

Uwineza, P.A.; Kwiatkowska, M.; Gwiazdowski, R.; Stępień, Ł.; Bryła, M.; Waśkiewicz, A. 2024. Field Assessment of *Lamium album* in Reducing Mycotoxin Biosynthesis in Winter Wheat Infected by *Fusarium culmorum*. *Agriculture*, 14, 647. https://doi.org/10.3390/agriculture14050647.

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Article

Field Assessment of *Lamium album* in Reducing Mycotoxin Biosynthesis in Winter Wheat Infected by *Fusarium culmorum*

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Abstract: Fungicides play a crucial role in conventional agriculture for disease control, but their prolonged use raises health and environmental concerns. *Fusarium culmorum* (*F. culmorum*), a major wheat pathogen causing Fusarium head blight (FHB) and Fusarium crown rot (FCR), poses significant mycotoxigenic threats. The application of natural plant extracts has been proven to fight against phytopathogenic fungi. This study aimed to a field experiment that was carried out at the Field Experimental Station of the Institute of Plant Protection—National Research Institute in Winna Góra, Poland, during the 2022/2023 season to evaluate the potential of *Lamium album* (*L. album*) flower extract as a foliar spray against mycotoxigenic fungi in two winter wheat varieties: Arkadia and Julius. The supercritical carbon dioxide extraction method (SC-CO_2) was employed to obtain the *L. album* flower extract. Ergosterol (ERG) and mycotoxin accumulation in the harvested wheat grains were analyzed using chromatography-based methods. The results demonstrated a notable reduction in ERG content in the field plots treated with *L. album* flower extract, from 26.07 µg/g (control group) to 8.91 µg/g (extract-treated group) for Arkadia and from 70.02 µg/g (control group) to 30.20 µg/g (extract-treated group) for Julius. The treatment with *L. album* reduced mycotoxin biosynthesis in both varieties, with deoxynivalenol (DON) and zearalenone (ZEN) production significantly decreased. Additionally, Arkadia exhibited greater resistance to *Fusarium* infection, and the antifungal effect of *L. album* was more pronounced than in the Julius variety, which proved to be more sensitive. In conclusion, *L. album* flower extract exhibited promising antifungal effects in field experiments to fight against *F. culmorum* in winter wheat varieties, suggesting a potential alternative to synthetic fungicides. However, as complete prevention of mycotoxin contamination was not achieved, further research is warranted to optimize extract concentrations and conduct long-term analyses to consider this plant extract as a sustainable control agent.

Keywords: winter wheat; natural plant extract; plant protection efficiency; sustainable agriculture; in vivo antifungal activity

Citation: Uwineza, P.A.; Kwiatkowska, M.; Gwiazdowski, R.; Stępień, Ł.; Bryła, M.; Waśkiewicz, A. Field Assessment of *Lamium album* in Reducing Mycotoxin Biosynthesis in Winter Wheat Infected by *Fusarium culmorum*. *Agriculture* **2024**, *14*, 647. https://doi.org/10.3390/agriculture14050647

Academic Editors: Isabel Lara and Claudia E. Moggia

Received: 16 March 2024
Revised: 10 April 2024
Accepted: 18 April 2024
Published: 23 April 2024

1. Introduction

Cereal grains play a vital role in the global economy as staple food for majority of the world's population, being a rich source of essential nutrients, including carbohydrates, proteins, fibers, vitamins, and minerals [1]. They contribute significantly to food security, economic well-being, and poverty alleviation. In 2022, global wheat production alone reached 808.4 million metric tons, underscoring the critical importance of cereals in meeting dietary needs [2].

However, cereal cultivation faces substantial threats from mycotoxigenic fungi, particularly those within the *Fusarium* genus. *Fusarium culmorum*, specifically, is responsible for causing two significant diseases in wheat: Fusarium head blight (FHB) and Fusarium crown rot (FCR) [3,4]. FHB is far more critical promoted by warm and humid environments, develops from infection at anthesis and spreads until grain harvest. Conversely, FCR is prevalent worldwide in arid and semi-arid cropping regions [5]. The frequency and extent of FHB and FCR epidemics have escalated due to climate change, conservation agriculture, and rising wheat-maize crop rotations [3]. These diseases threaten global wheat production, contributing to yield and quality losses that can adversely impact food security. With an estimated 60% increase in global wheat production required over the next three decades to meet the demands of a growing population, the urgency of addressing these issues becomes apparent [3]. Furthermore, *F. culmorum* infections are invariably accompanied by the synthesis of various mycotoxins, notably zearalenone (ZEN) and its derivatives zearalenone-14-sulfate (ZEN-14S) and βeta-zearalenol (β-ZOL) and alpha-zearalenol (α-ZOL), as well as trichothecenes. These trichothecenes encompass type B trichothecenes such as deoxynivalenol (DON), acetyl-deoxynivalenol (3-ADON and 15-ADON), and nivalenol (NIV) [6,7], which pose severe risks to human health, livestock, food safety, and the economy [8].

To address these challenges caused by mycotoxigenic fungi in cereals, various studies explored good manufacturing practices and biological or chemical interventions to protect wheat from fungal infection and mycotoxin biosynthesis [9]. However, no foolproof technique protects cereals fully against *Fusarium* infections [10,11]. In addition, synthetic fungicides, such as benzimidazoles (thiabendazoles and carbendazim) and methyl bromide, have been extensively employed to combat these pathogens and protect crops effectively. Using these compounds has drawbacks such as pathogen resistance development, harm to non-target organisms, and potential toxicity for animals [12–14]. Seeking an alternative approach to control *Fusarium* pathogens with minimal risks and pursuing sustainable agriculture, the application of natural products derived from plants (plant extracts and essential oils) has gained prominence due to their various active compounds, such as alkaloids, phenols, and terpenes that have shown different biological properties, including antifungal activities [15,16].

Plant extracts are effective in controlling the development of *Fusarium* spp. both during the growing season and in storage [17]. The mode of action of plant-based compounds against fungi depends on various factors like fungal strain, the profile and purity of bioactive compounds, the type of solvent used for extraction, and the conditions in which these compounds are used [18,19]. In our previous research, extracts derived from *L. album* flowers have demonstrated efficacy in inhibiting the growth of *Fusarium* pathogens and reducing mycotoxin biosynthesis on PDA and in wheat seedlings [20]. Many authors have studied and confirmed the *in vitro* effect of various plant extracts against *Fusarium* spp. [16,19,21–24]. A growing number of plant extracts have been studied and tested as natural plant protection products [25], including Thyme Guard®, derived from *Thymus vulgaris*; Timorex Gold® obtained from *Melaleuca alternifolia*; Milsana®, sourced from *Reynoutria sachlinesis*; and Owel®, an extract obtained from *Macleaya cordata* [26,27]. Additionally, botanical solutions like NeemPro® derived from *Azadirachta indica* A. Juss [28] and NeemAzal® powder containing SilicoSec and azadarachtin (0.1%) [29] have demonstrated efficacy, especially in post-harvest disease prevention and maize seed treatments. These available products highlight the potential for developing plant extracts to combat diseases caused by pathogenic *Fusarium* species, as the plant-based fungicide availability is still low. Most of the studies were conducted in vitro, so the in vivo experiments under field conditions are required, as successful effect of plant extracts to inhibit fungal growth in vitro does not guarantee its effect in field conditions.

Furthermore, the choice of extraction technique significantly influences plant extracts' quality and inhibitory effect. Traditional methods, including maceration and distillation [24,30], often involve prolonged extraction times and large solvent quantities, raising

regulatory concerns. In contrast, the shift towards green solvent extraction, particularly supercritical fluid extraction (SFE) using CO_2 (SC-CO_2), addresses these limitations. The application of SFE for obtaining various natural compounds has been extensively evaluated [31–33], which aligns with sustainability goals as it utilizes non-toxic, non-flammable CO_2 under high-pressure and lower-temperature conditions. This method yields extracts with distinct chemical complexities compared to conventional techniques, making it a promising and environmentally-friendly choice for plant material extraction [31,34].

In the pursuit of sustainable agriculture, the main goal of this research was the assessment of antifungal potential of *L. album* flower extracts obtained through SC-CO_2 to protect winter wheat Arkadia and Julius cultivars against *F. culmorum* and associated mycotoxins biosynthesis in field conditions.

2. Materials and Methods

2.1. Plant Material and Extraction

Dried *L. album* flowers were purchased from a certified Polish company called Dary Natury in Podlaskie Voivodeship of Poland. Plant material was extracted using a dynamic-static extraction lab scale, as described by [19]. Multiple vessels were prepared, each containing ten grams of powdered *L. album* flowers, and then placed in an oven set at 50 °C/250 bar. The CO_2 flow rate was maintained at 4 mL/min, with methanol as a fixed co-solvent at 1 mL/min. Each extraction run lasted 180 min. The process continued until a sufficient volume of *L. album* extract was obtained. Subsequently, obtained extracts were evaporated in a vacuum evaporator (Buchi Labortechnik AG, Flawil, Switzerland) at 40 °C to eliminate methanol; dried extract was then reconstituted in a measured volume of distilled water (10:2, *v*/*v* ratio) and stored at −18 °C until required for the antifungal assays.

2.2. Studied Material

The studied material consisted of two commonly used winter wheat cultivars varying in susceptibility to *Fusarium*: a susceptible cultivar Julius and a resistant cultivar Arkadia. Both cultivars originated from the Plant Breeding Company in Poznań, Poland.

2.3. Fusarium Strain and Inoculum Preparation

Fusarium culmorum strain KF 846 was obtained from the collection of the Institute of Plant Genetics, Polish Academy of Sciences in Poznan, Poland. The pathogen was cultured in Petri dishes (9 cm diameter) containing potato dextrose agar medium (PDA, BioShop, Burlington, ON, Canada) and incubated in the dark at 28 °C for seven days to promote fungal growth. Upon maturation, fungal spores were harvested by washing the culture plate with sterile water, gently scraping the surface to dislodge the spores, and then filtering the suspension through sterile cheesecloth to remove any mycelial debris. The concentration of spores per milliliter was determined using a hemocytometer and adjusted to achieve a final concentration of 700,000 spores/mL.

2.4. Chemicals

Carbon dioxide (CO_2, SFE grade), contained in a dip tube cylinder, was purchased from Air Products (Poznan, Poland). Methanol for HPLC-super gradient was purchased from POCh (Gliwice, Poland). Acetonitrile, methanol, and water for LC-MS grade were acquired from POCh (Gliwice, Poland). Analytical standards purchased in ready-to-use solutions from Romer Labs (Tulln, Austria) included ZEN, DON, and 15+3-AcDON at 100 µg/mL. The β-ZOL concentration was 10 µg/mL. ZEN-14S (100 µg/mL) was purchased in Aokin (Berlin, Germany). ERG (a fungal growth indicator) was purchased from Sigma-Aldrich (Steinheim, Germany).

2.5. Experimental Design and Procedure

The field experiment was carried out in the 2022/2023 growing season at the Field Experimental Station of the Institute of Plant Protection—National Research Institute in

Winna Góra, Poland (52°12′41.7″ N, 17°25′45.6″ E). The experiment was set up in a random block design with four repetitions on plots of 3.75 m^2 with two cultivars of winter wheat: Arkadia and Julius. On 6 June 2023, in the BBCH 63 phase (flowering), inoculation with a spore suspension of *F. culmorum* was performed. A backpack sprayer was used for inoculation, applying (at a working pressure of 0.2 MPa) 170 mL of a suspension with a concentration of 700,000 spores/mL to the plot once. The day after inoculation (7 June 2023), *L. album* extracts were applied using a backpack sprayer. In the case of plots where plant extracts were used, the flow rate of the working liquid was 125 mL/plot (at a working pressure of 0.3 MPa). At maturity (4 August 2023), grains were harvested with a plot harvester Wintersteiger, model Classic (Ried, Austria), and prepared for analysis.

2.6. Weight of 1000 Grains Evaluation

Grain samples were randomly selected for analysis. Each sample plot was divided into three batches, each comprising 200 grains. Subsequently, the individual batches were weighed. The average weight of 1000 grains were computed using the mean weight obtained from the three batches with the purpose of assessing the impact of *Fusarium* infection and the efficacy of *L. album* treatment on the grain quality. Results are expressed in grams.

2.7. Chemical Analyses

2.7.1. ERG Content in the Harvested Wheat Grains

Harvested wheat was ground into a fine powder and then analyzed for the ergosterol content following the method described by Waśkiewicz et al. (2014) [35]. Briefly, wheat samples (100 mg) were suspended in 2 mL of methanol in a culture tube, and 0.5 mL of 2 M aqueous sodium hydroxide was added. The mixture underwent three 10 s microwave irradiations (370 W) and was then neutralized with 1 mL of 1 M aqueous hydrochloric acid. After treatment, samples were subjected to extraction with n-pentane (3 × 4 mL), and collected extracts were evaporated to dryness using a stream of nitrogen. The resulting dry residues were reconstituted in 1 mL of methanol and filtered through a syringe filter of 0.2 µm mesh. Using a chromatographic system, ERG separation was carried out on a 3.9 mm Nova Pak C-18 (Waters, Milford, MA, USA), 4 mm column, employing methanol: acetonitrile (90:10, *v*/*v*) as the mobile phase at a 1.0 mL/min flow rate. Detection of ERG was performed using a Waters 2996 Photodiode Array Detector (Waters, Milford, MA, USA) set at 282 nm. ERG was quantified by measuring the peak areas at the retention time according to the relevant calibration curve. The limit of detection was 10 ng/g.

2.7.2. Mycotoxins in the Harvested Winter Wheat Grain

Mycotoxins were extracted by adding a mixture of 20 mL acetonitrile, water, and formic acid (79:20:1, *v*/*v*/*v*) to 5 g of fine powder of winter wheat grain samples. Extraction process involved vortexing for approximately 30 s and mixing on a horizontal shaker for 24 h, samples were centrifuged at 7500 rpm for 10 min. Afterwards, approximately 2 mL of mycotoxin extracts were filtered through a 0.20 µm nylon syringe filter and collected in vials for mycotoxin identification and quantification.

Mycotoxins detection and determination were analyzed with UHPLC-HESI-MS/MS. The analytes were separated on a non-porous C18 Cortecs chromatographic column (100 mm × 2.1 mm × 1.6 µm). The mobile phase consisted of water–methanol 90:10 (A) and methanol–water 90:10 (B); both phases had 5 mM ammonium formate and 0.2% formic acid. The following flow gradient (A/B ratio) was applied: 100:0 for 0–2 min; 75:25 for 2–3 min; 40:60 for 3–6 min; 0:100 for 6–20 min; 0:100 for 20–26 min; 100:0 for 26–28 min; 100:0 for 28–30. The flow rate was 0.3 mL/min, and sample volume (2 µL) was injected into the system [19].

The compounds were quantitatively analyzed using multiple reaction monitoring. All samples were analyzed in triplicate.

2.8. Statistical Analysis

In this study, statistical analyses were conducted using Analysis of Variance (one-way ANOVA model) to compare mean mycotoxin levels, ergosterol content, and thousand wheat grain weights among different treatment groups (control vs. extract) for both Arkadia and Julius wheat varieties. Post hoc analyses were performed using the Duncan multiple range test (Statistica 13.3. StatSoft, Tulsa, OK, USA) to identify specific pairs of groups with statistically significant differences. The significance level was set at $p < 0.05$, ensuring a robust statistical evaluation. The experiment was done in four replications.

3. Results

3.1. Comparative Effect of L. album Flower Extract on the Grain Weight of the Harvested Winter Wheat Cultivars

The results presented in Table 1 depict the treatment effect of *L. album* on the weight of harvested wheat grains (1000 wheat kernels) for both Arkadia and Julius cultivars that have been artificially infected with *F. culmorum*.

Table 1. The effect of *L. album* on the weight of harvested wheat grains by comparing the studied cultivars.

Plot *	ARK_C	ARK_E	JUL_C	JUL_E
FUS 1A-405	43.30 ^{a,*} ± 0.18	43.65 ^{a} ± 0.09	38.65 ^{c} ± 0.10	41.45 ^{b} ± 0.17
FUS 1A-302	43.25 ^{a} ± 0.15	44.10 ^{a} ± 0.04	38.30 ^{c} ± 0.06	39.15 ^{c} ± 0.19
FUS 1A-204	44.00 ^{a} ± 0.21	44.40 ^{a} ± 0.03	37.80 ^{c} ± 0.19	38.2 ^{c} ± 0.31
FUS 1A-103	44.05 ^{a} ± 0.08	43.70 ^{a} ± 0.26	35.80 ^{d} ± 0.03	36.05 ^{d} ± 0.01

* According to Duncan's test, different letters correspond to significant differences ($p < 0.05$) between means. ARK_C: Arkadia control; ARK_E: Arkadia treated with extract; JUL_C: Julius control, JUL_E: Julius treated with extract. Plot *: symbolize the plot numbers used for the repetitions.

Regarding the Arkadia cultivar, the control group yielded wheat grains with an average weight of 43.25 to 44.05 g. The weight was marginally enhanced in the *L. album*-treated group, ranging from 43.65 to 44.40 g. The overall mean weight for the control group was 43.65 g. However, the extracts-treated group had a slightly higher average weight of 43.95 g. Within this cultivar, there were no significant differences observed between the control and extract-treated groups across all plot numbers, as indicated by the lack of variation in letter labels.

In contrast, for the Julius cultivar, notable differences in the grain weights were observed between the control and extract-treated groups. In general, the control group consistently showed lower mean grain weights (37.65 g) compared to the extract-treated group that showed a mean grain weights of 38.70 g. This trend was consistent across all plot numbers and was statistically significant, as denoted by the distinct letter labels assigned to the mean values.

In overall, the results suggest that the application of *L. album* flower extract has a positive effect on the weight of harvested wheat grains, particularly evident in the Julius cultivar.

3.2. ERG Level in the Harvested Wheat Grains

Analyzing ERG levels in harvested wheat grains from the studied cultivars, Arkadia, and Julius, infected with *F. culmorum* and treated with *L. album* extract, was crucial for assessing fungal biomass (a fungal growth indicator) and the extract's antifungal efficacy. Comparing ERG levels between treated and untreated samples helped in evaluating the extract's effectiveness in inhibiting fungal growth. Additionally, it provided insights into potential variations in susceptibility to *Fusarium* infection among different wheat cultivars. Therefore, the results showed that applying *L. album* as a natural antifungal agent against *F. culmorum* in winter wheat cultivars has led to a differential reduction in ERG content

for both cultivars (Table 2). In the case of the Arkadia cultivar, the control group exhibited ERG levels ranging from 18.64 to 35.92 µg/g, with an average of 26.07 µg/g. Conversely, samples treated with *L. album* extracts demonstrated a significant decrease in ERG content, ranging from 5.63 to 13.01 µg/g, with an average of 8.91 µg/g.

Table 2. The effect of *L. album* on ERG content [µg/g] of the two winter wheat varieties.

Plot *	ARK_C	ARK_E	JUL_C	JUL_E
FUS 1A-405	28.50 ^{f,*} ± 0.76	5.63 ^{i} ± 0.62	53.54 ^{c} ± 2.25	35.62 ^{e} ± 3.69
FUS 1A-302	21.25 ^{g} ± 0.97	13.01 ^{h} ± 2.67	46.61 ^{d} ± 2.76	29.26 ^{f} ± 1.17
FUS 1A-204	18.64 ^{g} ± 1.16	7.40 ^{i} ± 1.66	63.75 ^{b} ± 3.83	20.88 ^{g} ± 0.97
FUS 1A-103	35.92 ^{e} ± 4.35	9.60 ^{hi} ± 1.25	116.17 ^{a} ± 6.62	35.02 ^{e} ± 1.98
Mean	26.07	8.91	70.02	30.20

* According to Duncan's test, different letters correspond to significant differences ($p < 0.05$) between means. ARK_C: Arkadia control; ARK_E: Arkadia treated with extract; JUL_C: Julius control, JUL_E: Julius treated with extract. Plot *: symbolize the plot numbers used for the repetitions.

Similarly, in the Julius cultivar the control group displayed ERG content ranging from 46.61 to 116.17 µg/g, with an average of 70.02 µg/g. The extract-treated group exhibited a noticeable reduction in ERG content, ranging from 20.88 to 35.62 µg/g, with an average of 30.20 µg/g. Consequently, the results affirm that *L. album* extracts effectively reduced ergosterol content in both wheat varieties, underscoring their antifungal properties.

Notably, the Arkadia cultivar demonstrated a more substantial reduction in ergosterol, marking a 65.82% decrease compared to the control group. In contrast, the Julius cultivar exhibited a 56.86% reduction in ERG relative to the control groups. These differential impacts of *L. album* extracts on ergosterol reduction, highlight the potential variations in cultivar response to fungal infection and the antifungal treatment. Specifically, Arkadia proved its resistance to *F. culmorum* compared to Julius, which exhibited greater susceptibility.

3.3. The Inhibitory Impact of L. album Flower Extracts on Mycotoxin Biosynthesis in the Investigated Winter Wheat Cultivars

The ability of *L. album* extracts to inhibit and reduce the mycotoxin accumulation in winter wheat cultivars artificially inoculated with *F. culmorum* was assessed. The presence of DON, 3- and 15-acetyl-deoxynivalenol (3- + 15-AcDON), ZEN, zearalenone-14-sulfate (ZEN-14S), α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) was analyzed in both control and extract-treated groups (Table 3). The data were statistically analyzed using ANOVA and post-hoc tests (Duncan test) with a significance level of $p < 0.05$.

The produced mycotoxins were the same in both varieties. However, the levels of mycotoxins accumulated in the harvested grains were significantly different, with the control group exhibiting the highest concentration of mycotoxins compared to the *L. album*-treated samples. The accumulation of the mycotoxins in both cultivars was statistically different, with the Julius cultivar showing more mycotoxin accumulation compared to the Arkardia. Additionally, among the biosynthesized mycotoxins in both cultivars, ZEN-14S (285.71–668.97 µg/g) was the most produced, while β-ZOL (1.09–2.94 µg/g) and α-ZOL (0.26–3.53 µg/g) were the least produced. In the Arkadia cultivar the mycotoxin accumulation was as follows: ZEN-14S > ZEN > 3+15AcDON > DON > β-ZOL > α-ZOL. For the Julius cultivar it was different: ZEN-14S > ZEN > 3+15AcDON > DON > α-ZOL > β-ZOL. Furthermore, Figure 1 highlights the potential of *L. album* in reducing mycotoxins in both cultivars, with the Arkadia cultivar exhibiting a more substantial reduction in mycotoxin levels, ranging between 33.29 to 46.82%, compared to the Julius variety, where mycotoxins were reduced in the range from 22.34 to 40.66%, with DON and ZEN the most reduced mycotoxins in both varieties.

Table 3. Mycotoxins produced [μg/g] by *F. culmorum* KF 846 strain in the absence/presence of *L. album* extracts in Arkadia and Julius winter wheat cultivars.

ARK_C						
Plot *	**DON**	**3- and 15-AcDON**	**ZEN**	**ZEN-14S**	**α-ZOL**	**β-ZOL**
FUS 1A-405	68.54 ^{f,*} ± 1.45	106.56 ^{e} ± 5.93	175.74 ^{c} ± 3.80	550.30 ^{d} ± 22.29	0.57 ^{g} ± 0.03	1.75 ^{ef} ± 0.04
FUS 1A-302	77.61 ^{e} ± 2.93	104.20 ^{e} ± 10.78	143.78 ^{e} ± 4.52	400.46 ^{e} ± 17.49	0.54 ^{g} ± 0.04	1.86 ^{de} ± 0.04
FUS 1A-204	45.91 ^{hi} ± 4.39	82.96 ^{f} ± 5.29	105.06 ^{i} ± 8.54	338.01 ^{f} ± 43.19	0.27 ^{g} ± 0.02	1.03 ^{gh} ± 0.19
FUS 1A-103	52.04 ^{h} ± 7.81	111.64 ^{e} ± 17.29	134.92 ^{fg} ± 1.80	524.45 ^{d} ± 24.89	0.46 ^{g} ± 0.03	2.84 ^{bc} ± 0.18
Mean	61.02	101.34	139.88	453.30	0.46	1.87
ARK_E						
Plot *	**DON**	**3- and 15-AcDON**	**ZEN**	**ZEN-14S**	**α-ZOL**	**β-ZOL**
FUS 1A-405a	33.14 ^{j} ± 3.77	55.41 ^{g} ± 3.99	65.38 ^{l} ± 4.42	392.24 ^{e} ± 22.95	0.42 ^{g} ± 0.05	0.39 ^{i} ± 0.05
FUS 1A-302a	43.40 ^{i} ± 3.11	79.04 ^{f} ± 1.49	97.52 ^{ij} ± 4.05	215.79 ^{g} ± 7.60	0.24 ^{g} ± 0.03	1.10 ^{gh} ± 0.12
FUS 1A-204a	29.83 ^{jk} ± 2.44	54.86 ^{g} ± 4.40	67.48 ^{l} ± 4.61	131.50 ^{h} ± 26.86	0.14 ^{g} ± 0.03	0.89 ^{hi} ± 0.23
FUS 1A-103a	23.45 ^{k} ± 2.09	79.90 ^{f} ± 3.89	80.87 ^{k} ± 1.39	403.30 ^{e} ± 11.98	0.30 ^{g} ± 0.06	1.96 ^{de} ± 0.29
Mean	32.46	67.30	77.81	285.71	0.27	1.09
JUL_C						
Plot *	**DON**	**3- and 15-AcDON**	**ZEN**	**ZEN-14S**	**α-ZOL**	**β-ZOL**
FUS 2A-405	98.37 ^{bc} ± 3.41	161.44 ^{b} ± 9.82	186.91 ^{b} ± 9.72	733.00 ^{b} ± 11.18	2.16 ^{e} ± 0.17	1.85 ^{de} ± 0.35
FUS 2A-302	84.77 ^{d} ± 4.45	128.91 ^{d} ± 3.06	142.19 ^{ef} ± 5.26	510.65 ^{d} ± 58.76	4.04 ^{b} ± 0.53	2.90 ^{b} ± 0.13
FUS 2A-204	104.84 ^{b} ± 3.87	145.63 ^{c} ± 5.43	188.80 ^{b} ± 3.50	805.38 ^{a} ± 9.01	5.24 ^{a} ± 0.70	2.86 ^{bc} ± 0.63
FUS 2A-103	135.04 ^{a} ± 3.22	195.30 ^{a} ± 6.88	222.23 ^{a} ± 6.45	626.84 ^{c} ± 48.48	2.67 ^{d} ± 0.24	4.15 ^{a} ± 0.53
Mean	105.76	157.82	185.03	668.97	3.53	2.94
JUL_E						
Plot *	**DON**	**3- and 15-AcDON**	**ZEN**	**ZEN-14S**	**α-ZOL**	**β-ZOL**
FUS 2A-405a	45.31 ^{hi} ± 3.91	127.82 ^{d} ± 2.49	128.90 ^{g} ± 2.69	520.88 ^{d} ± 13.73	1.16 ^{f} ± 0.38	1.29 ^{fgh} ± 0.20
FUS 2A-302a	61.88 ^{g} ± 5.42	78.89 ^{f} ± 2.94	94.02 ^{j} ± 3.80	340.10 ^{f} ± 40.91	3.36 ^{c} ± 0.49	2.33 ^{cd} ± 0.37
FUS 2A-204a	49.14 ^{hi} ± 4.03	106.04 ^{e} ± 5.93	118.23 ^{h} ± 3.07	381.46 ^{ef} ± 32.81	4.35 ^{b} ± 0.19	1.48 ^{efg} ± 0.43
FUS 2A-103a	94.68 ^{c} ± 3.59	154.44 ^{bc} ± 5.59	160.01 ^{d} ± 2.46	609.29 ^{c} ± 12.52	1.64 ^{f} ± 0.26	3.28 ^{b} ± 0.33
Mean	62.75	116.80	125.29	462.93	2.62	2.10

* According to Duncan's test, different letters correspond to significant differences ($p < 0.05$) between means. Analyzed mycotoxins: deoxynivalenol (DON), 3- and 15-acetyl deoxynivalenol (3- and 15-AcDON), zearalenone (ZEN), zearalenone-14-sulfate (ZEN-14S), alpha-zearalenol (α-ZOL), and beta-zearalenol (β-ZOL). ARK_C: Arkadia control; ARK_E: Arkadia treated with extract; JUL_C: Julius control, JUL_E: Julius treated with extract. Plot *: symbolize the plot numbers used for the repetitions.

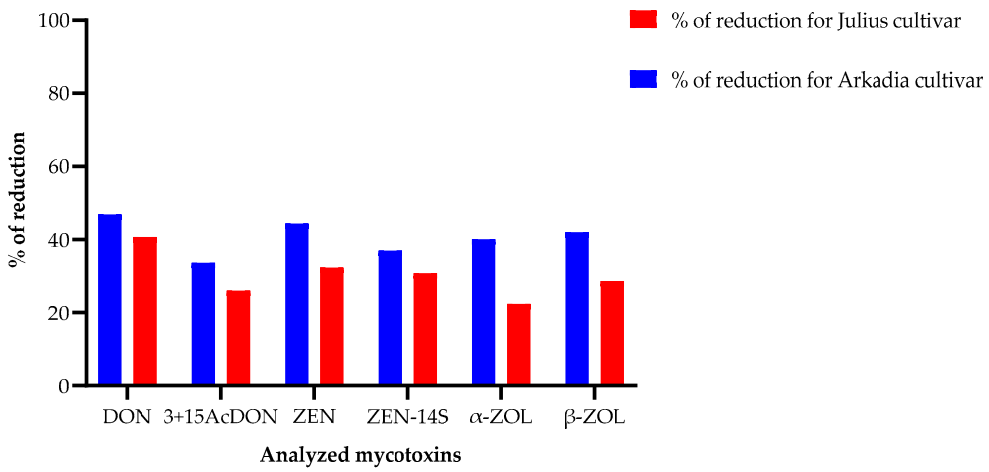

Figure 1. Comparative effect of *L. album* on mycotoxin reduction in winter wheat cultivars Arkadia and Julius. Data are expressed as % of mycotoxins reduction. Analyzed mycotoxins: deoxynivalenol (DON), 3- and 15-acetyl deoxynivalenol (3- and 15-AcDON), zearalenone (ZEN), zearalenone-14-sulfate (ZEN-14S), and beta-zearalenol (β-ZOL) and alpha-zearalenol (α-ZOL).

4. Discussion

Winter wheat is a major global crop [36] but is vulnerable to *Fusarium* infections, particularly FHB, which poses significant threats to grain yield, germination, quality, and safety [6]. Controlling *Fusarium* diseases in wheat is crucial due to the absence of complete resistance in any wheat variety, the long-term survival of the pathogen in soil, and the production of harmful mycotoxins in wheat tissues, which are harmful to consumers. To mitigate these issues and reduce reliance on chemical pesticides, there is growing interest in using natural plant extracts as protective agents. Many plants and their extracts have demonstrated antifungal properties [19,22,23,37,38].

While there is a growing interest in biological control methods, significant emphasis is placed on understanding and screening different plant species and natural compounds that can control fungal growth and mycotoxins in vitro. However, the expanding knowledge in this area should be used more extensively for practical applications in planta, pre-harvest, post-harvest, as well as during storage and food processing. To our knowledge, no previous research under field conditions has investigated the antifungal effect of *L. album* flower extracts. Additionally, studies of other natural plant extracts against *Fusarium* infection in winter wheat cultivars are still limited, and there is an increasing demand for organic produce [39]. The current study builds upon our prior research, conducted under controlled conditions, which has demonstrated the protective efficacy of *L. album* against artificially inoculated *F. culmorum* in wheat seedlings [40]. The present results confirm the capability of *L. album* to inhibit fungal growth under field conditions, as evidenced by the ERG and mycotoxins reduction in the harvested wheat grains and a moderate difference in thousand-kernel weight.

Specifically, Arkadia exhibited a higher weight, measuring 43.65 g in the control group and 43.97 g in the extract-treated group. Conversely, Julius cultivars displayed lower weights, with 37.65 g in the control group and 38.70 g in the extract-treated group. Some studies emphasize significant varietal differences, while others report no notable distinctions. For instance, a study assessing new winter wheat cultivars for organic farming found no discernible difference in 1000-kernel weight between Arkadia (41.6 g) and Julius (41.2 g) despite variations in other morphological features and canopy parameters influencing their competitive abilities [41]. This trend is further supported by an investigation examining wheat varieties' response to seed cleaning and fungicide treatment following FHB infection. The study noted variations in thousand-kernel weight attributed to wheat variety, with SY Wolf (28.6 g) and Everest (28.4 g) displaying higher weights compared to WB Grainfield

(27.2 g) [42]. This means that 1000-kernel weight alone may not be a decisive factor in determining the effect of plant extract, resistance, or yield potential of wheat varieties.

The observed decrease in ERG levels, a crucial component of fungal membranes, represents a significant finding in our study. The application of *L. album* extract resulted in a substantial decrease in ergosterol content, with a 65.82% reduction for the Arkadia cultivar and 56.86% for the Julius cultivar, indicating a potential disruption in fungal cell membranes, contributing to the inhibitory effect on *F. culmorum* growth in both cultivars. The inhibitory effect of *L. album* extract on *F. culmorum* growth in both cultivars could be attributed to its active compounds, such as verbascoside; isoscutellarein derivatives; flavonoids (quercetin, quercetin-3-O-glucoside, rutin, isoquercitrin, kaempferol-3-O-glucoside); and phenolic acids (protocatechuic, chlorogenic, vanillic, and caffeic), along with phenylpropanoid glycoside ester derivatives (lamalboside, acteoside, and isoacteoside) that have been identified with various biological activities [43–47]. The detrimental impact on ERG content indicates a significant disruption or blockage in biosynthesis within the plasma membrane of *F. culmorum*. This finding is consistent with previous studies that reported decreased ERG content in *Fusarium*-infected samples after applying plant extracts. For instance, the *Solanum torvum* Swartz leaf extract reduced ERG production in *Fusarium verticillioides* [48]. Additionally, thymol, a component of plant extracts, has been shown to inhibit the growth of *F. graminearum*, resulting in a reduction of ERG levels [49]. Similarly, extracts derived from *Melissa officinalis* demonstrated a concentration-dependent reduction in ERG content [19]. Furthermore, assessing ergosterol levels in harvested wheat grains after *F. culmorum* infection is crucial for identifying the susceptibility and resistance of studied cultivars to *Fusarium* infection. The observed reduction in ergosterol aligns with the antifungal activity of *L. album* extract, emphasizing its efficacy in impeding *F. culmorum* growth.

Although ERG is a reliable predictor of fungal development, it is not a dependable indicator of mycotoxin contamination [19], as not all fungi synthesize mycotoxins. Conversely, mycotoxins can persist even in the absence of fungal viability. In the present study, the application of *L. album* extract (obtained by SC-CO_2) in the form of foliar spray exhibited mycotoxins reduction (DON, 3- + 15-AcDON, ZEN, α-ZOL, β-ZOL, and ZEN-14S) by 22.34 to 46.82% relative to control samples and a significant reduction was observed in DON and ZEN levels in both varieties. These findings are in line with other studies, demonstrating the effect of the extracts from medicinal and aromatic plants on *Fusarium* species [37,50–52]. Abbas and Yli-Mattila (2022) showed that methanolic extract of the medicinal plant *Zanthoxylum bungeanum* successfully decreased *F. graminearum* growth and abrogated DON production in wheat heads [51]. Similarly, mustard-based extracts reduced fungal infection in wheat grains and decreased DON accumulation under growth chamber conditions but were ineffective under field conditions [52]. Furthermore, the natural extracts from neem seeds demonstrated more potent inhibition of mycotoxin production of DON, 15AcDON, 3AcDON, and ZEN [38].

The α-ZOL (0.26–3.53 µg/g) and β-ZOL (1.09–2.94 µg/g) were the least produced mycotoxins, while ZEN-14S (285.71–668.97 µg/g) was the highest among the produced mycotoxins in both cultivars (Table 3). This also gives insight into the types of mycotoxins and their levels that were biosynthesized by the inoculated strain of *F. culmorum*, and it confirmed that natural plant extracts may have an inhibitory effect to several mycotoxins at once. ZEN-14S, α-ZOL, and β-ZOL are the modified forms of ZEN identified in this study. They are currently unregulated but may increase the risk of human animal exposure because of possible high incidence in cereals and cereal-based products, serving as staple food in several regions [53]. Veršilovskis et al. (2019) mentioned that ZEN-14S is approximately 60 times more estrogenic than ZEN and can be readily hydrolyzed to ZEN in the gastrointestinal tract, thereby increasing exposure to ZEN [54]. Furthermore, a high ZEN-14S/ZEN ratio in the malted wheat suggests that *Fusarium* can convert ZEN into a phase II metabolite through sulfation reactions [55]. Ayed et al. (2011) evaluated the cytotoxicity and genotoxicity of α-ZOL and β-ZOL in vivo, in mouse bone marrow cells and in vitro, in cultured HeLa cells, and compared it with ZEN. Their results showed that

ZEN and α-ZOL exhibited the same range of genotoxicity and cytotoxicity; both were more genotoxic and cytotoxic than β-ZOL [56].

In the present study, Arkadia was found to be more resistant to *F. culmorum* infection as it exhibited a lower ergosterol content and mycotoxin accumulation than the Julius cultivar, which was more sensitive and exhibited a high amount of ergosterol and mycotoxin accumulation as the fifth category for classifying wheat resistance (resistance to mycotoxins) [57]. This supports the notion that inherent differences may influence the susceptibility to fungal infection and the extract's effectiveness in combating *Fusarium.* Our findings align with the existing literature, reinforcing the significant varietal differences in susceptibility to *Fusarium* infection and mycotoxin accumulation in winter wheat cultivars [41,57,58].

5. Conclusions

Biological control and the search for new natural antifungal agents are gaining more and more interest. The potential of *L. album* flower extract in combatting *F. culmorum* in winter wheat cultivars was proven by reduced ERG, DON, and ZEN in the harvested wheat grains of both winter wheat cultivars Arkadia and Julius, compared to the controls. These results highlight the extract's promising role in promoting sustainable and resilient agricultural practices, contributing to the broader global discourse on eco-friendly disease management strategies. Furthermore, different mycotoxins were identified in the control and extract-treated groups. The results showed the most minor production of α-ZOL and β-ZOL, with ZEN-14S being the most abundant mycotoxin in both cultivars. This underscores the importance of considering multiple mycotoxins and their modified forms in evaluating the efficacy of natural plant extracts. To our knowledge, this is the first report showing the in vivo possibility of using *L. album* as a natural antifungal agent for some devastating winter wheat plant diseases. This study contributes valuable insights in using *L. album* flower extracts for sustainable and eco-friendly *Fusarium* control in winter wheat. The observed cultivar differentiation highlights the need for tailored approaches, and our findings pave the way for further exploration of natural plant extracts in promoting sustainable wheat production and valorizing locally available plants.

Author Contributions: Conceptualization, P.A.U. and A.W.; methodology, P.A.U., M.B., R.G. and A.W.; validation, P.A.U. and A.W.; formal analysis, P.A.U., M.K. and M.B.; investigation, P.A.U., M.K. and A.W.; resources, Ł.S., R.G. and A.W.; data curation, P.A.U. and A.W.; writing—original draft preparation, P.A.U., and A.W.; writing—review and editing, P.A.U., Ł.S. and A.W.; visualization, P.A.U.; supervision, A.W.; project administration, P.A.U. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Polish National Science Centre, grant number 2018/31/B/NZ9/03485.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Data is available upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

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